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13. ABSTRACT ***(Maximum 200 Words)***

In order to fulfill the need for a simple self-contained device for the detection of *Yersinia pestis* target DNA, we combined unique approaches that exploited solid phase methods for DNA extraction, isothermal amplification and visual detection. In Phase I of this project, optimization of DNA extraction and purification was achieved using a unique capture system, Xtra Bind™. A homogeneous method of isothermal strand displacement amplification was used in the first part of this program to amplify a 572 base pair PCR product insert of the *caf1* gene. A detection method using lateral flow immunochemistry was adapted to the detection of the amplified products. By September 1999, we demonstrated the feasibility of all the elementary steps within the device. In the Phase II of this project, new primer sets were designed for *Y. pestis* genomic DNA and the best set optimized for low limit of detection. Extensive changes were made to the extraction, amplification and detection chemistries in order to achieve our limit of detection in human plasma samples. In addition, changes in the design of the device were made in order to render it functional. A level of 1 X 10^3 starting copies of target was achieved within the device.

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John G. Cardis 11-5-96

PI - Signature **Date**

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INTRODUCTION

Plague is classified as a re-emerging disease. The causative agent, *Yersinia pestis* is transmitted by fleas. Three virulence plasmids are characteristic in *Y. pestis*. These vary in different strains by size and genetic content. The pPCP1 plasmid codes for a plasminogen activator and pCD1 is a low calcium response plasmid. The largest of the three plasmids, pMT1 (60 -110 kB) is species specific and thus absent in *Y. enterocolitica* or *Y. pseudotuberculosis*[1]. This plasmid encodes a number of regulatory factors and a cluster of genes classified as a pathogenicity island that cumulatively participate in production of virulence factors.

In this program, we combined a unique approach that exploited solid phase methods for the nucleic acid extraction, amplification and detection[2]. The isothermal method of strand displacement amplification[3] (SDA) was used in the first part of this program to amplify a 572 base pair PCR product insert of the caf1 gene cloned into Invitrogen's pCR II vector. These efforts were important first steps in the development of a detection system for *Y. pestis* DNA and resulted in a high level of sensitivity and precision.

In the second phase of this project, SDA was applied to specifically detect the caf1 gene of *Y. pestis* chromosomal DNA. Extensive changes were made to the extraction, amplification and detection chemistries in order to achieve our projected limit of detection in human plasma samples. In addition, we made substantial changes in the design of the SCIP device in order to efficiently transfer our robust system to a self-contained platform.

MATERIALS

Solid Phase for extraction
Xtra Bind™ or Xtra Amp™ (Xtrana, Inc.) was used for all solid phase extractions

Lysis Buffer
The lysis buffer was composed of 1N NaOH, 2X SSC, 1 M Urea, 15 mM $CaCl_2$, 100 mM Tris-HCl, 8% PEG 8000, 0.5 % Triton X-100, pH 11.0

Wash Buffer
The wash consists of 10 mM Tris HCl pH 8.0, 150 mM LiCl, and 1 mM EDTA

SDA Reagents
Buffer system
The buffer system chosen was a phosphate buffer system (BD WIP# 360658). It contained the co-solvents dimethyl sulfoxide (DMSO) and glycerol (BD WIP# 360644)

Enzymes
The *Bso*BI and *Bst* DNA Polymerase Large Fragment enzymes were obtained from New England Biolabs (*Bso*B1 586-L Special 900; *Bst* DNA Polymerase 275-L Special 900).

Primers
Primers consisted of a pair of SDA primers (S-primers) and a pair of bumper primers (B-primers). These were designed using OLIGO™ Version 5.0 software (National Biosciences, Inc., Plymouth, MN.). Primers were obtained from Operon, Biosource International or IDT; all were polyacrylamide gel electrophoresis (PAGE) purified.

Detection Probes
Two detection probes were used for the amplified product detection. One detection probe was labeled with FITC molecule at the 5'-end and phosphorylated at the 3'-end . The second probe was labeled with biotin at the 3'-end. The probes were purchased as HPLC-purified oligonucleotides from Operon, Biosource International, or IDT.

Nucleotides
dNTPs used were supplied by BD Biosciences: dGTP(BD WIP# 328810), dATP(BD WIP# 330440), dTTP(BD WIP# 330572) and dCsTP(BD WIP# 330546).

Additional SDA reagents
Additional components: Bovine serum albumin (BSA) (BD WIP# 360653), human placental DNA (BD WIP# 360645), and magnesium acetate (BD WIP# 360647)

DNA
Y. *pestis* cloned plasmid and genomic DNA were obtained from Capt. David R. Shoemaker, Ph.D., and Fred Knuert, Ph.D., Diagnostics Systems Division, Ft. Detrick, MD.

Plasma
For target sample preparation, *Y. pestis* genomic DNA was spiked into human plasma obtained from healthy blood donors by Bonfils Blood Center, Denver. Plasma was collected in either sodium citrate or EDTA treated BD Vacutainers.

LATERAL FLOW COMPONENTS

Nitrocellulose
The nitrocellulose selected for this work was a large pore direct cast nitrocellulose with a polyester backing (Millipore) with a capillary rise of 75-100 sec/4 cm DI H_20.

Sample Pad: #237 (Ahlstrom)

Conjugate Release Pads
Glass fiber #9254 (Lydall Technical Papers) or AccuFlow™ P and G (Schleicher & Schuell) were used as release pads throughout this work.

Microparticle Conjugates
Streptavidin (International Enzymes, Inc., Fallbrook, CA) was conjugated to blue carboxylate-modified microparticles (Seradyn, Inc., Indianapolis, IN.) with 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide-HCl (Pierce, Rockford, IL) using a standard in-house conjugation procedure.

TNGA Buffer
25 mM TRIS, pH 8.4, 100 mM NaCl, 1% Fish Skin Gelatin (FSG), containing 0.1 % NaN_3 (TNGA Buffer)

Absorbent Medium
Wicking was facilitated with bonded cellulose acetate (Transpad™ wicks R-18552, Filtrona Richmond Inc., Richmond, VA), cellulose absorbents, (VWR 320, Ahlstrom, Mt. Holly Springs, PA) and #470, ¾" paper (Schleicher & Schuell).

Adhesive
Acrylic pressure sensitive adhesive laminate, supported with 74 lb. white polypropylene coated silicone release liner (0.010 inch, GL-187; G&L Precision Die Cutting, San Jose, CA)

Striper
The linear reagent dispensing system consisted of the Striper/Digispense 2000 System (IVEK Corporation, North Springfield, VT). The Striper Controller was typically set for a rate of 40 mm/sec for the dispensing of the anti-FITC IgG [$F(ab)'_2$ fragment of affinity purified goat anti-Fluorescein antibody (Rockland, Gilbertsville, PA)]. The Digispense 2000 Controller was set at a dispense rate of 4.0 µl/sec.

Shear
Nitrocellulose membranes were consistently cut into 2.5 - 3.0 mm x 25 mm strips with the Matrix 2360 Programmable Guillotine Shear (Kinematic Automation, Twain Harte, CA). In some cases the cutting was achieved manually.

Laminator
The Matrix 2210 Universal Laminator Module (Kinematic Automation) was used to laminate absorbent pads, nitrocellulose and conjugate release pads. In some cases lamination was achieved manually.

METHODS

DNA extraction:

Solid Phase for Extraction

The analysis and characterization of Xtra Bind™ was critical to the development of this program. X-ray diffraction (XRD) and Fourier Transform Infrared spectroscopy (FTIR) analyses assisted us in the selection of the best material to be used in the capture of genomic DNA from *Y. pestis*. Appendix III describes some of the important features of this reagent.

Extraction Buffer Optimization

Aseptic techniques were used throughout the procedure. Two capture systems were used while optimizing the conditions for *Y. pestis* DNA extraction and amplification. During initial characterization of the basic extraction buffer components, we used Xtra Amp™ tubes (Xtrana, Inc.) and experimentally selected concentrations of *Y. pestis* target DNA. For these analyses, target DNA was mixed with diluted human plasma and an equal volume of lysis buffer. The mixtures were incubated in Xtra Amp tubes at room temperature for 30 minutes after which the target mixture was removed. The tubes were washed two times with Xtra Amp Wash Buffer and the remaining bound target DNA was amplified selectively by SDA. Modifications to this nominal extraction buffer were made by following a standard protocol for experimental design using Design-Expert software (Stat-Ease) and monitoring the impact of buffer additives on target DNA binding to Xtra Amp tubes.

Final modifications to the extraction buffer were made by evaluating the efficiency of target DNA binding to a slurry of Xtra Bind™. In these reactions, 10 µl of Xtra Bind™ (vol/vol) was added to a plasma:extraction buffer mixture (1:2) that was spiked with purified *Y. pestis* DNA. Binding of the target DNA proceeded at room temperature on a rotating wheel for 20 minutes. The reaction tubes were centrifuged briefly to sediment the Xtra Bind™ material and the unbound DNA was removed by aspiration. Xtra Bind™, with bound target DNA, was washed two times with 500 µl of Kit Wash Buffer and the resulting material was transferred to a clean PCR tube. Forty microliters of SDA Master Mix was added to each tube and equilibrated with the Xtra Bind™ material. Following denaturation of the target DNA, and cooling of the mixture to 53°C, 10 µl of enzyme mixture was added and the amplification reaction proceeded for 60 minutes at 53°C.

STRAND DISPLACEMENT AMPLIFICATION (SDA)

Principle

Strand displacement amplification (SDA) is a bi-phasic isothermal amplification process consisting of target generation and exponential amplification phases. SDA uses a restriction enzyme and a polymerase to exponentially amplify specific DNA targets. The restriction enzyme *Bso*BI and the exonuclease deficient polymerase, *Bst* DNA Polymerase (Large Fragment), can achieve amplification factors of 10^{10} in less than 0.5 hr. This system is driven by the sequence specific nicking action of *Bso*BI and the action of *Bst* DNA polymerase as it extends from the nick, displacing a strand of double stranded target into solution. The extension reaction reconstitutes the nickable restriction site by incorporation of a phosphorothioate deoxynucleotide and the cycle repeated.

Procedure

The methodology used here represents a slightly modified version of the amplification procedure described by Walker et al. (1992). Primer sets for use in the SDA reaction were designed using Oligo version 5.0 primer analysis software (Molecular Biology Insights, Cascade, CO). The reaction buffer and enzyme concentrations were optimized using the Design-Expert program (Stat-Ease) and the conditions were established initially using target molecules in aqueous reactions. The reaction conditions were finalized and validated for application in the SCIP device by amplification of DNA molecules bound either in Xtra Amp tubes or to a slurry of Xtra Bind™. SDA reactions were performed in 50µl reactions containing (final concentrations) 500 nM SDA primers (S-primers), 50 nM Bumper primers (B-primers), 50 mM K_2HPO4, 5% DMSO, 100 µg/ml BSA, 710ng of human DNA, 5 mM $MgOAC_2$, 9.1% glycerol, 1mM dATP, dTTP, dGTP, and 7 mM [S]-dCTP. Target DNA molecules were denatured by incubating reaction tubes at 95°C for two minutes in a heat block followed by rapid cooling to 53°C. Amplification was initiated by the addition of 80 Units BsoBI and 18.9 Units Bst DNA polymerase, large fragment (New England Biolabs), and the amplification products were analyzed after 1 hour incubation at 53°C by lateral flow, as described.

Homogeneous SDA

Homogeneous SDA was identified as an important step required to achieve the complete integration of SDA chemistries in the SCIP device. Our approach involved the incorporation of detection probes in the SDA reaction mixture (35 nM FITC labeled probe and 35 nM of biotin labeled probe). Here, 50µl SDA amplified product was detected immediately following the amplification by simply exposing the lateral flow strip laminates to the amplified product. Product dilution and heat denaturation were not necessary in this case.

SDA Primer Design

The following guidelines were followed:

1. Mismatches with the target sequence towards the 5' end of the primer were avoided
2. GC, GG, CG and CG clamps at the 3' end of the primers were avoided
3. Primers with high internal stability at the 3' end were avoided
4. Stretches of the same nucleotide sequence were avoided

Precision Studies

Precision of the SDA reaction was evaluated by conducting replicates of 5 amplifications on different days. Figure 7 summarizes the results of this study

STATISTICAL EXPERIMENTAL DESIGN

In order to accelerate the optimization processes for this project we used statistical experimental design[4,5]

LATERAL FLOW DETECTION

Principle

Biotinylated and fluoresceinated oligonucleotide detection probes were added to the SDA reaction mixture after amplification. At this time, if the cafl gene target was present, the two sets of probes specifically hybridized with the same strand of the target DNA. When mixed with the Streptavidin-coated dyed microparticles, the biotinylated portion of probe bound to the Streptavidin. The resultant complexes were then applied to a nitrocellulose membrane containing an immobilized anti-Fluorescein isothiocyanate antibody line. Migration of the complexes occurred through the membrane until the antibody bound the fluoresceinated portion of the probe. This interaction arrested further migration of the microparticle-haptenized duplexes and rapid accumulation of the complexes occurred at the antibody line. The unaided eye easily detected this result.

Procedure

A typical reaction employed the detection probe mix (DPM) consisting of 0.54 μM fluoresceinated detection probe (DPF) and 0.18 μM biotinylated detection probe (DPB) were diluted in water. Streptavidin-coated microparticles were diluted in TNGA buffer to a final concentration of 0.18% solids. Anti-FITC lateral flow strips (3 mm x 25 mm) were used throughout the study.

Five microliters of DPM was mixed into 50 μl of SDA amplified product and the mixture placed in a 95°C heat block for 2 minutes. After cooling to 23°C ± 3°C, 5 μl of 0.18% solids SA-MPs was added and the suspension mixed thoroughly. After incubation at room temperature for 2 minutes, the LFT strips were placed into tubes and the complete reaction volume (60μl) was allowed to wick through the laminate. The final concentrations for the DPF and the DPB were 45 nM and 15 nM respectively, and the SA-MPs were at 0.160%. The results were recorded after approximately 5 minutes.

LFT LAMINATES FOR THE SCIP DEVICE

Laminates (Figure 1) were designed to optimize transfer of fluid from the reagent tube (Figure 8) to the nitrocellulose.

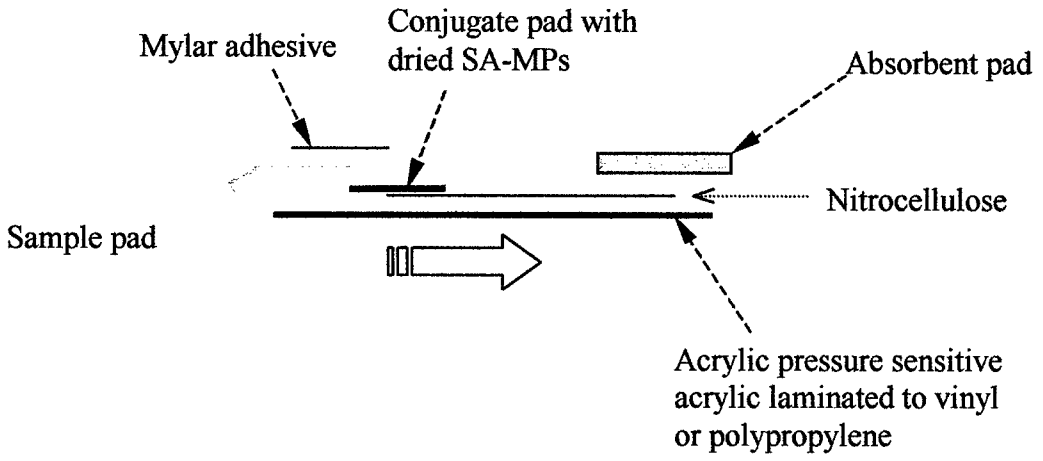


Figure 1. Schematic of a multi-component LFT assembly for SCIP. Block arrow indicates the direction of capillary flow. The conjugate pad containing the SA-MPs was omitted in earlier constructs where the homogeneous SDA was conducted in the presence of the SA-MPs.

The consistent and even transfer of liquid to the LFT laminate was investigated in the device. Experiments and modifications were designed to ensure reproducible transfer to the laminate.

Fluid transfer to laminate

SCIP devices were assembled with strips attached to waste tube rib using 4 drops of 3M pressure sensitive adhesive. Sample wicks protruded through the sealing surface (flange) at distances from 0 mm to 1.5 mm in order to study fluid transfer.

Mounting of laminate

Efforts were directed at reducing uneven flow rates that occurred due to the recruitment of fluid from the laminate to the edge of the laminate shelf. The laminate was moved slightly away from shelf of the inner waste tube in order to reduce adherence of fluid.

SCIP DEVICE ENGINEERING

Ease of Rotation of Reagent Tube

Easy rotation of the reagent tube was important for the proper functioning of the device. Precise engineering was critical to achieve this goal. To this end we lightly lubricated the aluminum alloy ring and the outside of the reagent tube with KRYTOX®.

Loss of Reactants to the Waste Reservoir

The loss of amplified product to the waste reservoir due to over-rotation of reagent tube was a major determinant of LFT strip dysfunction and poor reactivity. It was reasoned that the increasing the flange area of the waste tube through the use of a shim would resolve this problem. This modification prevented leakage of amplified product into the waste tube and away from the LFT strip. The materials evaluated for this purpose were Lexan, Delrin and Polypropylene (0.020" to 0.030").

Indexing

An indexing system was added to the reagent tube in order to make the relative operative positions of the device obvious to the user. Introducing three small notches on the outer and reagent tubes achieved this. The SCIP device was assembled and rotated, using the cap handle, from "waste", to "heat" and then to "elute".

Fluid Management: Transfer to the Waste Tube

The complete removal of reaction mixtures and wash solution from the reagent tube was important for the proper functioning of the SCIP device and changes were directed to the proper fluid management and containment. Absorptive media were evaluated for their flow characteristics and strength and it was important that the media made contact with the bottom of the screen in the reagent tube to initiate the wicking process. In the first attempts at absorbing the waste fluid, we used a very expansive absorbent material but various flow problems associated with this material prompted us to seek other media. We exploited the absorptive properties of glass fiber (GF) and cellulose, the media of choice in many diagnostic applications. The results were better absorption without swelling and rapid removal of waste fluid from reagent tube.

SCIP Assembly

SCIP devices were very carefully assembled. It was important to clean the reagent tube, screen and shim according to the standard protocol. Extra effort was taken to ensure that the surface of the shim was level. The 0-ring on the thermal ring was used as a guide to determine the uniformity of seal. The LFT laminate was assembled so that it was on its shelf but not in contact with the right angled portion of the waste tube (Figure 11). The devices were assembled in the "amplify" (heat) position to minimize movement of parts during shipment and the entire device was stored desiccated at $23^{\circ}C \pm 3^{\circ}C$.

Integration

The extraction step was integrated into the SCIP device by performing unit gravity flow. The Xtra Bind™ was placed in the reagent column of the device and the extraction components and washing buffer were added. The time required for complete flow was 2-5 minutes with minimal handling. The extraction done within the SCIP device, however, only detected 10^6 to 10^7 of *Y. pestis* target DNA. It was realized that further work would be necessary to increase the overall sensitivity.

The interfacing of the elementary steps into the SCIP device was done through extensive troubleshooting, optimizing and modifying both the chemistry and the device. The preliminary SCIP prototypes produced by Ansys Diagnostics had shown the feasibility of interfacing each of the chemistries separately; however, when all 3 steps of extraction, amplification, and detection were incorporated, leakage was a problem. Both Xtrana and Ansys engineers focused on the resolution of this problem. The cause of the reagent leakage through the silicone seal ring mounted on the aluminum thermal ring was investigated extensively and finally determined to be due to pressure differentials within the device. This problem was resolved by venting the device.

The original amplification scheme for the SDA reaction was modified to improve the interfacing of SDA with the SCIP device. The 95°C denaturation step prior to amplification was revisited and this step was successfully eliminated. In order to accommodate the SCIP device, a new approach involved preheating the SDA master mix followed by the direct addition to the extracted target at 53°C. Upon the addition of the preheated master mix, the temperature of the SDA reaction mixture was briefly increased to 80°C. This modified method of denaturation worked adequately for the amplification and detection of high copies of extracted target. Although the SCIP device was not completely contained, the chemistries were successfully integrated in the device. To validate the functional aspects of the device, several SCIP devices were evaluated.

MODIFICATIONS TO THE SCIP IN ORDER TO IMPROVE PERFORMANCE

Shim

In order to prevent damage to the silicone ring or other sealing part used for the reagent tube, sharp edges on the front of the shim were rounded. In addition, a triangular projection was introduced on the back of the shim in order to prevent movement during the rotation of the reagent tube from the wash step. Figures 12 and 13 are drawings of the respective parts.

Reagent Tube

An insert consisting of a modified inverted PCR tube and cap in which the cap was perforated to allow for fluid transfer was designed. This was an important feature to ensure that the SDA reagents did not make contact with the polypropylene in the original reagent tube as this material was identified as inhibitory to low copy detection. Figure 14 is a drawing of the modified reagent tube.

Polypropylene Interference Studies

During the second phase of this project, interference due to unidentified inhibitors present in the polypropylene molded parts was evident. In a classical experiment, aliquots of Master Mix or Enzyme were incubated separately in reagent tubes in the 53°C water bath for 15 minutes. The reagent was transferred to fresh PCR tubes containing 1 x 10^5 copies of *Y. pestis* genomic DNA. The SDA reaction occurred in a thermocycler, with addition of Enzyme following the denaturation step. Table 7 shows the effect on the SDA reaction following contact with the molded part. Attempts to block the inhibitory effect by preincubation of the reagent tubes with BSA were not effective (Table 8).

Further studies were conducted with the following polypropylene resins: Montell Pro-Fax SD-242, Montell Pro-Fax SR-857M, Montell Pro-Fax PD-702, Huntsman PP-P4G4K-038, Millbrandt PP and Equistar P-51S30V. These materials were introduced into SDA reactions directly or after washing with methanol or acetone. Table 9 summarizes the results.

RESULTS

Optimization of Extraction Conditions

Introduction

The design of the extraction buffer to be used in our system defined a crucial stage in the development process of the proposed device. These functions had to be accomplished with maximum efficiency but with minimal impact on subsequent processes related to amplification and detection. In essence, this buffer system was designed to retain each of these broadly defined qualities: 1) efficient lysis of the bacterial target cells, 2) complete solubilization of desired components, 3) maximal binding of nucleic acid to the capture matrix, and 4) complete removal of unwanted inhibitory material in a single washing step. Characterization of a buffer system designed to meet these specifications was accelerated through the use of the Design-Expert™ software described previously.

Definition of Critical Components of the Extraction Buffer

The formulation of the extraction buffer was initiated using the standard Kit Lysis Buffer supplied with Xtrana's Xtra Amp™ Blood Extraction System. While this standard buffer performed satisfactorily during preliminary experiments, some loss of sensitivity was sustained. We sought to define more critically the influence of the various components of this buffer on the binding and amplification reactions. Implicit in these analyses was the view that the buffer composition would be sufficient to promote efficient lysis of bacterial cells. Our initial screening focused on the statistically significant contribution of each of the following: pH, Saline Sodium Citrate (SSC), Urea, NaOH, and $CaCl_2$. The results shown in Table 1 and Figure 2 provide an example of our initial screening procedure. Buffers were prepared according to formulations defined by the Design Expert program.

Also, in this example, incubation time was included as an additional parameter. The buffers examined in this block of experiments were adjusted to either pH 8 or pH 11. The results obtained following several preliminary experiments indicated that the pH of the solution made only a minor contribution to the required binding process. However, we observed greater reproducibility using buffers with higher pH values, leading us to establish pH 11 as the standard when evaluating other buffer components. An extensive analysis of greater than forty different buffer formulations, and their impact on nucleic acid binding and SDA performance, resulted in identification of several buffers that retained superior qualities in both required activities.

Run	pH	SSC	Urea	NaOH	$CaCl_2$	Incubation time	LF Result
1	8	1	0.5	0.8	5	30	2.0
2	11	1	1.0	0.8	5	10	1.0
3	8	3	0.5	0.2	15	30	1.3
4	8	1	1.0	0.8	15	10	2.5
5	11	3	1.0	0.2	15	10	1.5
6	8	3	1.0	0.2	5	10	1.3
7	11	1	0.5	0.2	15	10	0.5
8	8	3	0.5	0.8	15	10	1.3
9	8	3	1.0	0.8	5	30	3.0
10	11	3	1.0	0.8	15	30	3.8
11	11	3	0.5	0.8	5	10	1.8
12	11	1	1.0	0.2	5	30	1.3
13	8	1	1.0	0.2	15	30	0.8
14	11	1	0.5	0.8	15	30	2.8
15	8	1	0.5	0.2	5	10	0.5
16	11	3	0.5	0.2	5	30	2.0

Table 1. Design Expert Analysis of Extraction Buffer Components. All reactions were performed in Xtra Amp tubes. The reaction tubes were hydrated with 50 µl of extraction buffer and binding was initiated by addition of 50 µl of plasma/target DNA mixture. *Y. pestis* target DNA was present at 5 x 10^4 copies in the plasma/target mixture. Binding occurred during a static incubation at 25°C for the times indicated. Lateral Flow (LF) results are presented as an average of four replicate samples.

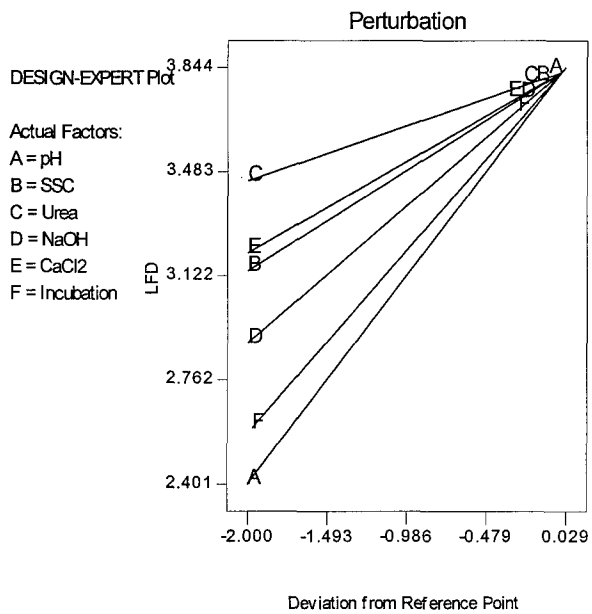


Figure 2. Perturbation plot obtained from Design Expert program indicates that the previous two level factorial results show that higher levels of all 6 factors are most optimal.

In this example, we also considered the exposure time of the target DNA to the Xtra Bind™ solid phase in the presence of each buffer, and the impact this variable would have on detection. At the minimum time tested, *Y. pestis* DNA could be detected following ten minutes incubation with Xtra Bind™ under several different conditions, although the amount of product that could be generated was lower than that observed following increased exposure time of target DNA to the solid phase.

A statistical analysis of the results shown in Table 1 indicated that in addition to incubation time, three factors exerted a significant influence on binding and amplification within the parameters defined by this experiment. The factors identified in this manner were SSC, Urea, and NaOH. We defined these three as the critical components that make up the standard extraction buffer and sought to better define their role in facilitating target capture onto Xtra Bind™ in the presence of human plasma. The outcome of this statistically designed analysis is shown in Table 2. As we had seen previously, several of the buffer formulations tested yielded inconsistent results. While these differences were not as dramatic as those encountered in several of our preliminary screenings, only those buffers that performed consistently were chosen for further study. Several of these were screened repeatedly for an ability to reproducibly promote target DNA capture and provide an active template for amplification. One buffer that consistently retained the desired qualities of efficient binding and robust amplification over a broad range of solid phase exposure time was selected as the final formulation that would make up our extraction/binding buffer. The critical components identified through statistical analysis and direct experimentation were the following: 2X SSC, 1M Urea, 1N NaOH, and 15 mM $CaCl_2$.

pH	SSC	Urea	NaOH	$CaCl_2$	Incubation time	LF Result
11	2	1	0.6	15	20	1.0
11	4	1	1.0	15	40	2.0
11	4	1	0.6	15	20	1.5
11	3	1	0.8	15	30	1.3
11	2	1	1.0	15	20	4.0
11	4	1	0.6	15	40	1.5
11	4	1	1.0	15	20	3.5
11	2	1	0.6	15	40	3.5
11	2	1	1.0	15	40	3.8
11	3	1	0.8	15	30	2.5
11	3	1	0.8	15	30	2.0

Table 2. Secondary Analysis of Extraction Buffer Critical Components. Various extraction buffers, defined by the Design Expert Program, were prepared and adjusted to pH 11. Plasma samples were spiked with 5 x 10^4 copies of *Y. pestis* genomic DNA and mixed with the test extraction buffers. Samples were incubated with the binding matrix for the indicated times and bound product was detected by SDA.

Coincident with this buffer refinement, we undertook a broader analysis of exposure time (Table 2). The minimum incubation time in this series, twenty minutes, was enough to bind sufficient target to generate detectable product. Additional incubation time was not always correlated with increased product generation. Binding kinetics was determined using an *in vitro* method that incorporated the Xtra Bind™ slurry and either static or rotating incubation. Although a positive response resulted following several incubation time points, the minimum binding period that still yielded maximal signal intensity was observed following 20 minutes of incubation of target DNA with the Xtra Bind™ slurry (Table 2). These experiments provided preliminary indication as to how the fluid sample flow would need to be regulated for efficient recovery to occur. A set of experiments similar to those shown in Table 3 was designed to identify an incubation temperature optimum; the goal being to complete as many sequential steps as possible at a single temperature. We were successful in defining ambient temperature as optimal for both the extraction process and nucleic acid binding. Thus, prior to the initiation of the SDA reactions, all manipulations could be performed at room temperature (data not shown).

We considered the possibility that one or more components could be added to our standard extraction buffer that would promote efficient lysis of bacterial cells and enhance retention of amplifiable target DNA, yet retard non-specific binding of plasma proteins. The results shown in Table 3 are representative of the types of compounds that were evaluated. Of the numerous compounds assessed, polyethylene glycol (PEG) and Triton X-100 were shown to provide a beneficial effect. Although not directly visualized in these experiments, an indication of their positive effect could be seen following dilution of the amplification products. Ten-fold dilutions of the products generated when binding was performed in the presence of either PEG or Triton X-100 were still detectable by lateral flow analysis when samples prepared with standard lysis buffer had reached endpoint dilution.

Run	**Reagents**	**Conc. (%)**	LF Result
1	PVP	0.5	3.5
2		0.1	2.8
3		0.1	3.3
4	PEG	8.0	4.0
5		5.0	4.0
6		2.0	3.3
7	Dextran	8.0	2.8
8		5.0	2.3
9		2.0	1.5
10	Dextran Sulfate	8.0	1.0
11		5.0	1.5
12		2.0	2.3
13	Triton X-100	1.0	3.0
14		0.5	4.0
15		0.1	3.3
19	Std. Lysis Buffer	--	4.0

Table 3. Effect of Buffer Additives. Compounds were added to the standard buffer (pH 11) at the concentrations indicated and tested for their effectiveness related to target DNA binding and SDA reaction. Lateral Flow results are expressed as an average of four replicates for each test run (PVP, polyvinylpyrrolidone; PEG, polyethylene glycol, MW 8000).

In pursuit of a buffer composition that would provide the greatest benefit to detection of low copy *Y. pestis* DNA present in a plasma sample, we prepared extraction/binding buffers containing either PEG or PEG + Triton X-100 and compared their effectiveness to the previously described Standard lysis buffer. The beneficial effect of these additions was determined by assessing the limit of detection following capture of the *Y. pestis* DNA target in the presence of each of these buffers (Table 4, Figure 3)

Expt	Lysis Buffer	Target	LF Result
1	STD. LB	$5x10^3$ copies	4.0
2	STD. LB	$1x10^3$ copies	1.8
3	STD. LB	$5x10^2$ copies	1.8
4	LB + PEG	$5x10^3$ copies	3.8
5	LB + PEG	$1x10^3$ copies	4.0
6	LB + PEG	$5x10^2$ copies	2.0
7	LB + PEG + Triton X-100	$5x10^3$ copies	3.5
8	LB + PEG + Triton X-100	$1x10^3$ copies	4.0
9	LB + PEG + Triton X-100	$5x10^2$ copies	2.3

Table 4. Comparison of Extraction Buffers using Limit of Detection Analysis. Plasma was spiked with the indicated copy number of *Y. pestis* target DNA and mixed with one of the three buffers indicated. After binding to the solid phase for 20 minutes at room temperature, the efficiency of binding was assessed by SDA and lateral flow detection.

These "additives" were included in the revised extraction buffer and demonstrated a significant positive effect on the limit of detection of *Y. pestis* target DNA present in an experimental sample of human plasma. In its final formulation, the extraction/binding buffer is as follows: 2X SSC, 1 M urea, 1 N NaOH, 15 mM CaCl2, 8% PEG, and 0.5% Triton X-100. It should be noted that while this revised extraction buffer evolved throughout a rigorous selective process, its composition remains relatively simple. We anticipate that this simplicity will serve us well when the reagent is incorporated into the final phase II product either dried or lyophilized.

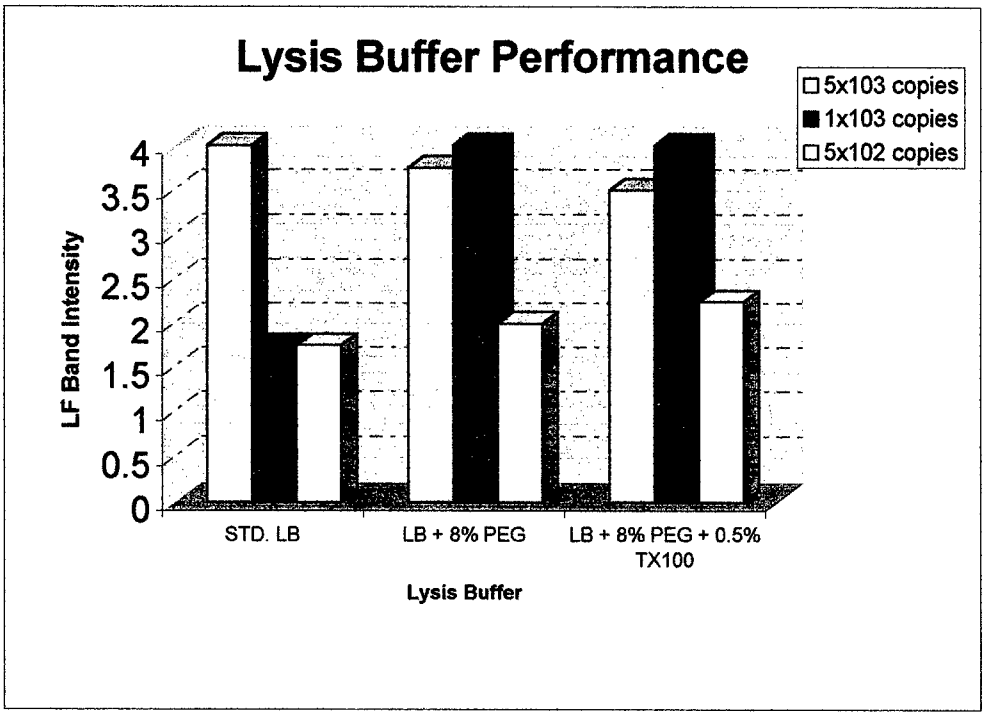
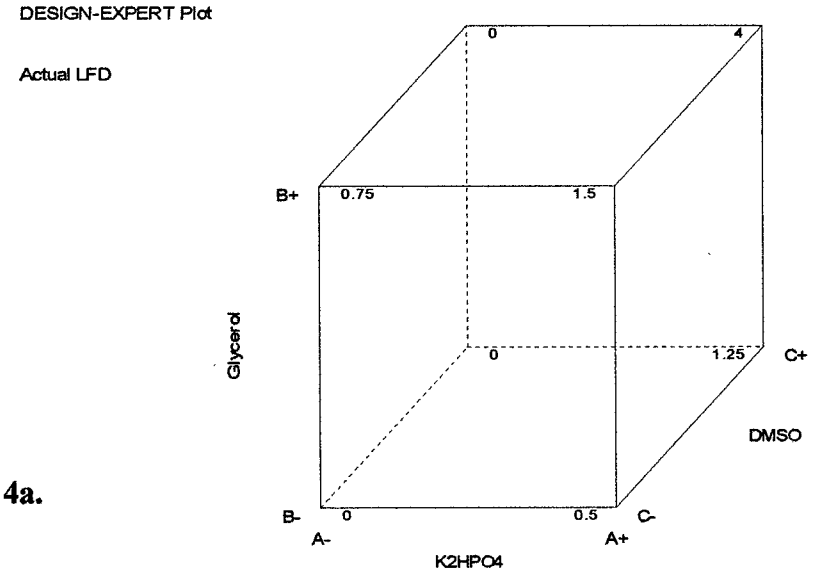


Figure 3. Limit of detection of final extraction buffer with and without various buffer additives. The addition of 8% PEG and 0.5% Triton X-100 significantly improved the performance of the extraction buffer. Target DNA was consistently detected at 500 copies.

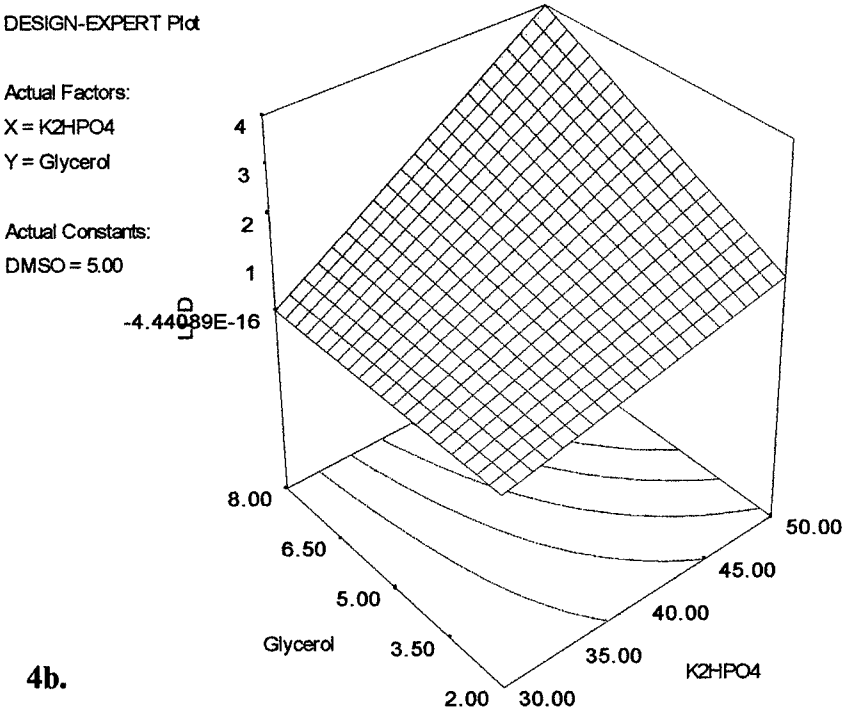
Optimization of Amplification with DoE: K_2HPO_4, Glycerol and DMSO

DoE was performed using 2-level factorial design with the three factors, K_2HPO_4, glycerol and DMSO. During that time we evaluated 4 primer sets and identified the one that generated the strongest and most robust signal at target levels near the LOD.

Figures 4a and 4b are cube and 3D surface response plots respectively, of the interaction between K_2HPO_4, glycerol and DMSO at a constant DMSO concentration of 5.0%. Higher signals were obtained at 50 mM K_2HPO_4 and 8% glycerol. This type of analysis enabled us to quickly determine hidden interactions that may have been missed if we were to perform single variable titrations.



4a.



4b.

Figures 4a and 4b. Cube and 3D surface response plots of the interaction between K_2HPO_4, glycerol and DMSO. Note that at a constant DMSO concentration (5.0%), the best signals are obtained with higher levels of K_2HPO_4 and glycerol.

Optimization of Amplification with DoE: Bst Polymerase and the BsoB1 Restriction Enzyme

The need to perform a DoE on the BsoB1 and Bst polymerase following the optimization of the K_2HPO_4, glycerol and DMSO was emphasized by collaborators at BD Biosciences. "Table 5" and Figure 5 are a two-way table and 3D surface response plot of the results.

"Table 5"

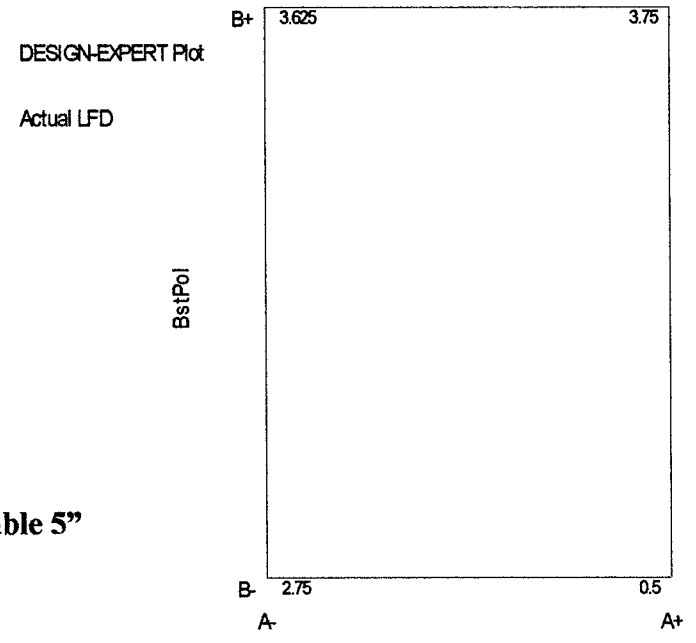


Figure 5.

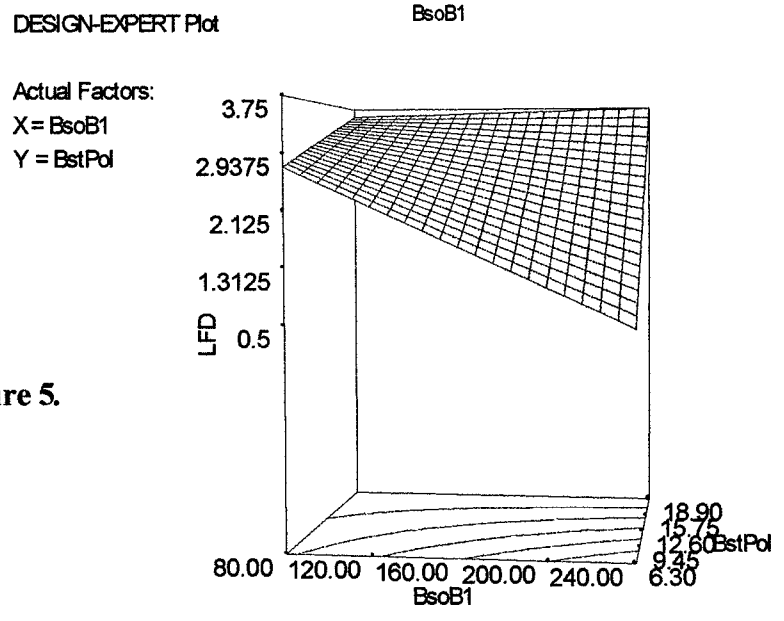


Table 5 and Figure 5. Two-way table and 3D surface response plot of BsoB1 and Bst polymerase. Note the wide range for optimum activity for BsoB1. Bst polymerase works best at higher enzyme levels.

The results of the analyses reflected in Table 5, Figure 5 and Table 6 shows the basis for proceeding with subsequent limit of detection studies.

The results suggested that the ratio of the enzymes was critical to achieving higher sensitivities in SDA. A BsoB1 and Bst polymerase ratio of 38 was clearly less efficient than ratios of 12.6 or less.

	BsoB1	**BstPol**	**$MgOAC_2$**	**Enzyme Ratio**	**$1X10^3$**	**$5X10^3$**	**$1X10^4$**	**Sum of reactions**
1	80	18.9	5	4.23	3.62	3.00	4.00	10.62
2	160	12.6	5	12.70	2.13	3.00	3.88	9.01
3	240	6.3	5	38.10	0.50	2.43	0.38	3.30
4	240	18.9	5	3.78	3.75	3.63	4.00	11.38
5	160	12.6	5	12.70	3.13	4.00	3.88	11.00
6	80	6.3	5	12.70	2.75	3.75	4.00	10.50

Table 6. Effect of the various combinations of *Bso*B1and *Bst* polymerase used in combination with three levels of target.

Limit of Detection Studies

The limit of detection achieved under the experimentally defined conditions is reflected in Figure 6.

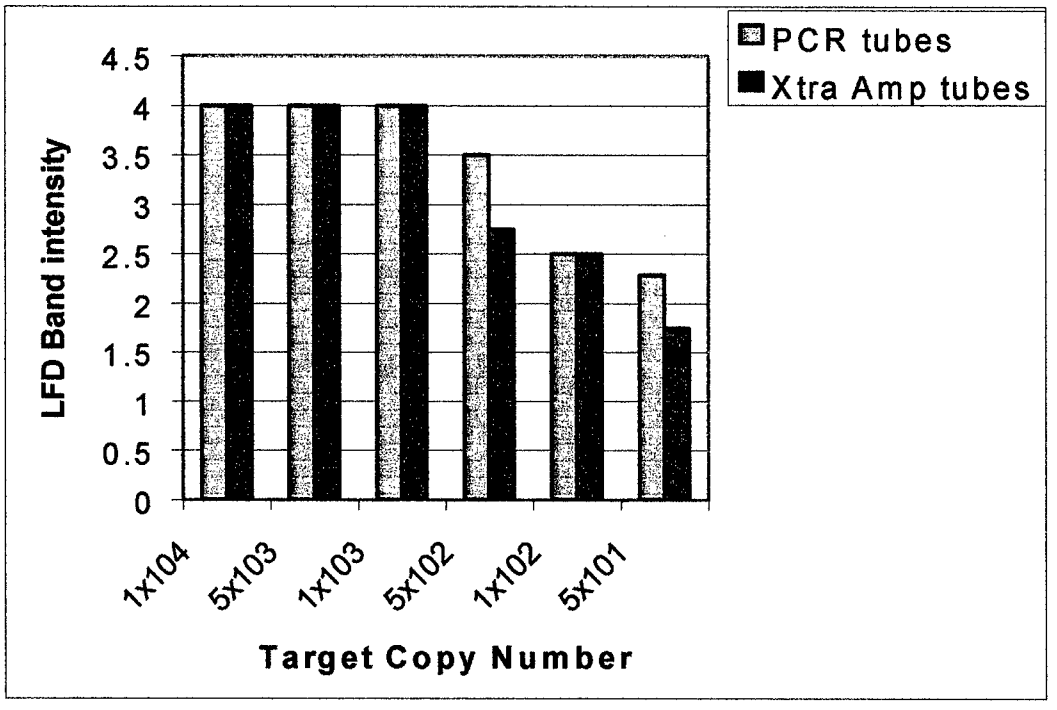


Figure 6. Response data for limit of detection studies. Comparative analysis of two amplification formats to validate statistically designed reaction buffer conditions and enzyme concentrations. Serial dilutions of purified *Y. pestis* genomic DNA were prepared in distilled water and added to reaction tubes as described in the text. Reactions were performed in either standard polymerase chain reaction tubes (PCR tubes) or Xtra Amp tubes. The amplified products were detected by Lateral Flow chromatography followed by visual evaluation of band intensity. Numerical designation of bands visualized on the lateral flow strips ranged from 0 to 4 and was based on comparison to results obtained with negative and positive controls, respectively (data not shown).

Precision Studies

The robustness of the optimized SDA conditions was evaluated by conducting a precision test using *Y. pestis* genomic DNA dilution. A level of 100 copies was detected with 100% precision (Figure 7)

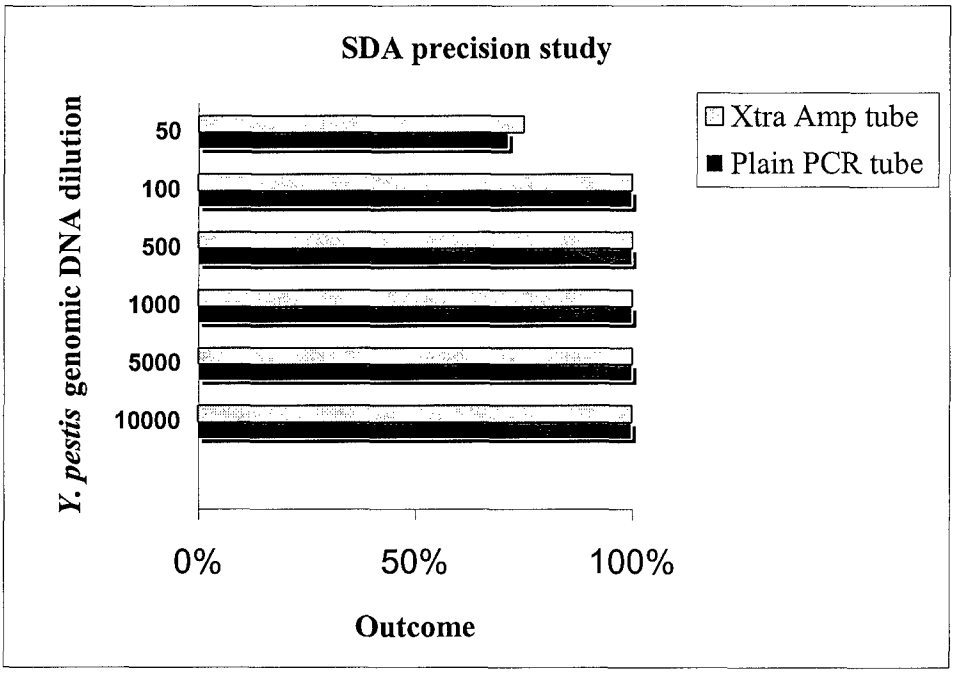


Figure 7. SDA precision study. *Y. pestis* DNA dilutions were amplified in replicates of eight in plain PCR tubes and Xtra Amp tubes. The sensitivity was 10^2 copies of target with 100% precision. Fifty copies were detectable 71-75% of the time.

SCIP ENGINEERING

The design and engineering of the SCIP device underwent constant changes to keep up with progress in the SDA chemistry. Figure 8 is a drawing of the cross section of the device used for the first part of this project.

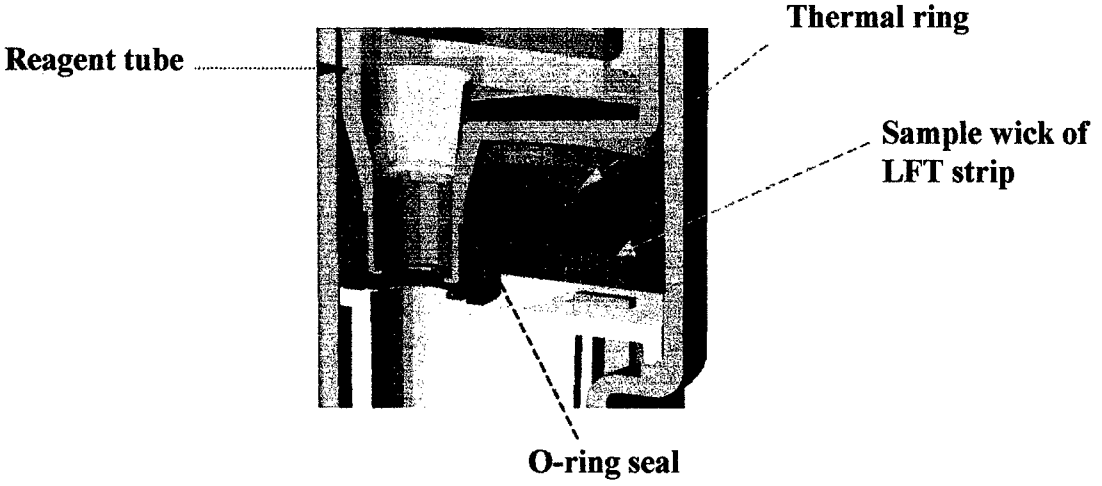


Figure 8. Schematic of a cross section on the essential components of the SCIP device (shown in the drain-to-waste position)

Rotation of Reagent Tube

Efforts to improve the ease of rotation of the reagent tube involved changes in the dimension of the tube and the use of a lubricant. Figure 9 illustrates the reagent tube and its immediate environment.

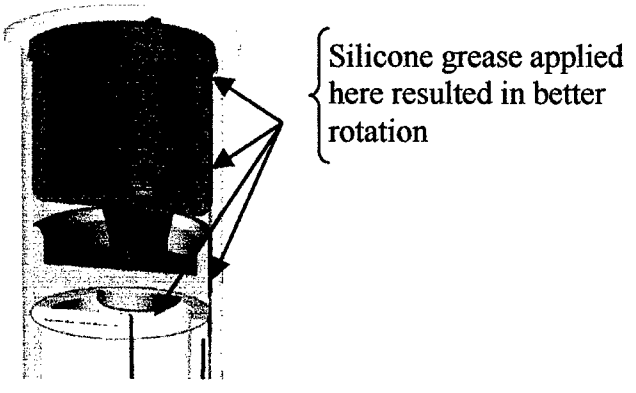


Figure 9. Regions of the SCIP device which were lubricated

Indexing
The correct positions for extraction, amplification and detection were not apparent in the first version and thus refinements were required at the design and molding levels. These changes subsequently resulted in a more reliable indexing system.

Fluid Transfer to the Waste Tube
We successfully wicked extraction and wash buffers to the waste tube reservoir with the aid of glass fiber (GF) absorbent & hydrophilic polyethylene nib inserted into the waste tube reservoir (Figure 10).

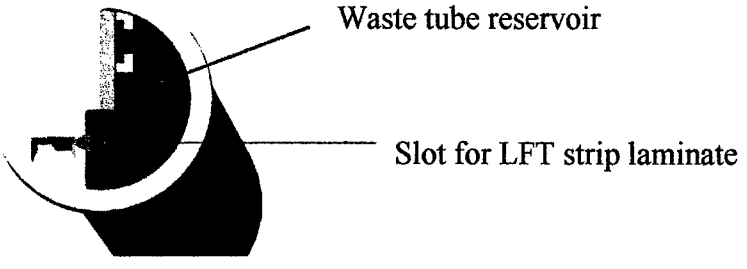


Figure 10. Portion of the inner tube showing the waste reservoir. The reservoir contains glass fiber absorbent & hydrophilic polyethylene nib.

GFs were selected because of their exceptional flow characteristics and strength. Furthermore, GFs can be enhanced by the addition of various binders (e.g. polyvinyl alcohol (PVA)) or wet strengthening agents (e.g. polyamide resins). Cellulose papers offer high purity, good liquid absorption and controlled wicking. The two materials were combined to produce optimum absorption and controlled flow rates.

Resolution of Over-Rotation of Reagent Tube and Loss of Reactants to the Waste Reservoir

The modification to the flange of the inner tube facilitated the reduction of the amount of waste evaporation and allowed for a larger seal area. Furthermore, the design appeared to improve containment of waste material and provide a better barrier between waste chamber absorbent and LFT strip (Figure 11).

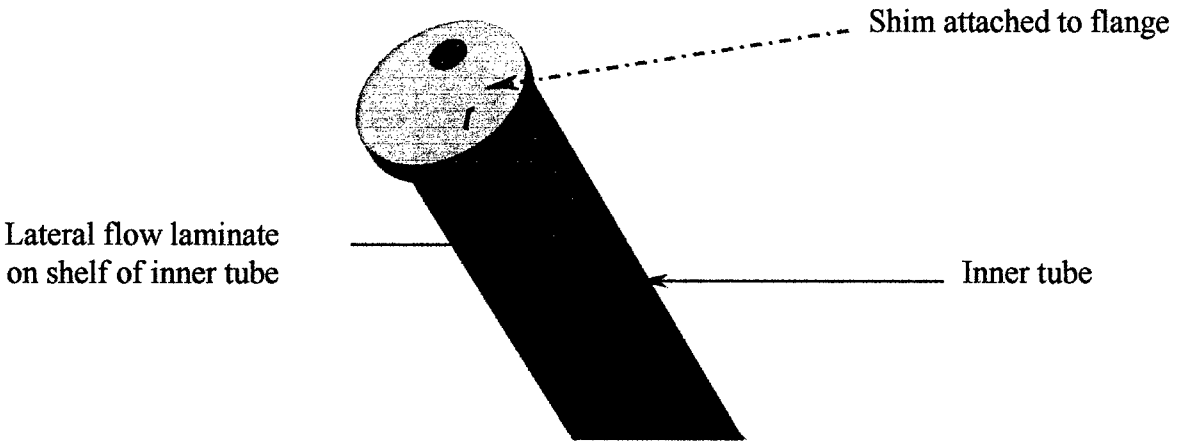


Figure 11. Modification of the flange surface by lamination with an additional material (shim)

This design, by virtue of its thickness, eliminated the need for a pre-load device (previously a silicon gasket) at the base of the inner and outer tubes. Evaluations conducted subsequently on the SCIP device suggested that the shim contributed to an overall improvement in performance of the device.

Mounting and Fluid Transfer to the Lateral Flow Test Strip in the Device
In early fluid transfer tests, uneven flow rates occurred due to recruitment of fluid from the LFT strip to the edge of the LFT shelf. Moving of the LFT strip away from shelf appeared to resolve this problem.

Modifications to the Shim

This component (Figures 12 and 13) was successful in reducing the transfer of moisture to the outer tube where the lateral flow strip was located. In addition, it provided a more reliable interface for the lateral flow strip. The chamfered edge of the lateral flow strip opening ensured that the silicone ring remained in tact thereby preventing product loss, and protected the strip from shearing during rotation of the reagent tube.

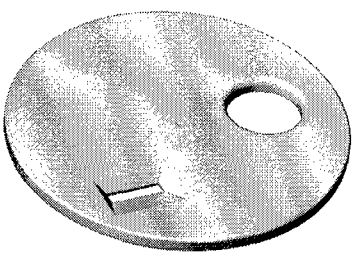


Figure 12. Top view of shim showing tapered modification in order to prevent rupture of silicone ring.

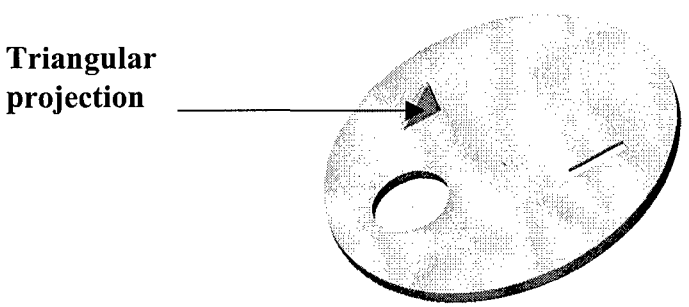
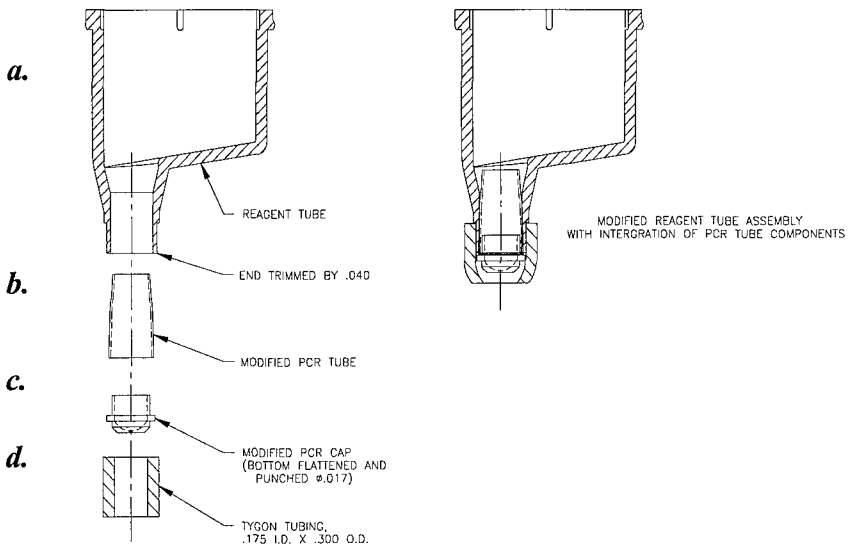


Figure 13. Bottom view of shim showing triangular projection that prevents movement of shim during rotation of reagent tube.

Modifications to the Reagent Tube

The modifications to the reagent tube (Figure 14) allowed for the successful amplification of the target DNA.



(I) Components of modified reagent tube **(II) Assembled reagent tube**

Figure 14. Schematic of the reagent tube components (I) and assembled reagent tube (II) destined for use in the SCIP device. The components shown above are *a.* the original reagent tube, *b.* a modified PCR tube, *c.* a perforated PCR tube cap and *d.* a cut Tygon tube.

General Appearance of the Modified SCIP Device

Although the rate of transfer of heat to the reaction chamber was reduced after the removal of the thermal transfer ring (Figure 15), the rate of heat transfer and the maintenance of the amplification temperature were satisfactory under these experimental conditions (Figure 16). These were confirmed by demonstration of amplified product in later experiments and in the successful demonstration on October 30, 2000.

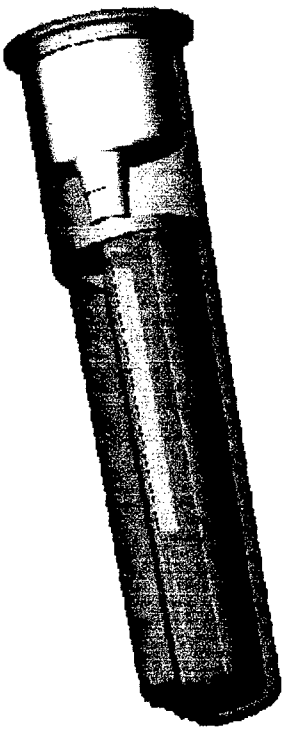


Figure 15. Drawing of the SCIP device without the thermal transfer ring.

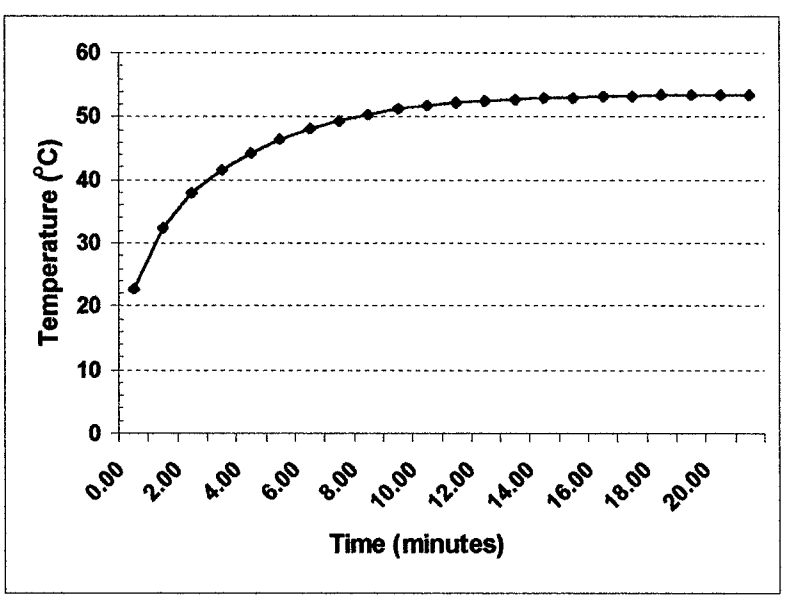


Figure 16. Rate of transfer of heat to the reaction area of the reagent tube.

POLYPROPYLENE INTERFERENCE STUDIES

The exposure of the SDA reagents to polypropylene revealed a consistent pattern of inhibition (Table 7).

Reaction conditions	Target DNA ($1x10^5$ copies)
MM + Enzyme	4
MM* + Enzyme	0
MM + Enzyme*	0
MM* + Enzyme*	0

Table 7. Example of inhibition of the SDA reaction with standard SCIP reagent tubes. Aliquots of Master Mix (MM) or Enzyme (*) were incubated separately in reagent tubes in the 53°C water bath for 15 minutes. The reagent (70 µl) was transferred to fresh PCR tubes containing 1 x 10^5 copies of freshly diluted *Y. pestis* genomic DNA. The SDA reaction occurred in a thermocycler, with addition of enzyme following the denaturation step.

Efforts to block the effect by prior exposure of the plastic to bovine serum albumin (BSA) did not prevent this effect (Table 8)

Reaction	Target DNA ($1x10^5$ copies)
BSA-coated tube	0
BSA-coated tube	0
MM + Enzyme/reagent tube	0
Control reaction in PCR tube	4

Table 8. Reagent tubes were incubated at room temperature with 200 µl of stock BSA diluted to 2mg/l. Incubation continued for 2 hours and the solution withdrawn. The target DNA was added (1 x 10^5 copies), followed by the addition of MM and Enzyme. The SDA reaction proceeded for 1 hour in the 53°C water bath.

Of special interest was the fact that polypropylene resins from several sources generally had no effect on the SDA reactions. When reagent tubes, molded from the respective resins were evaluated, the clear pattern of inhibition was again obtained (Table 9). While the source of this inhibitory was not identified, the modified SCIP design was functional.

Source & PP ID	Lateral Flow Test Results					Further information/ observations	Comments
	Resin			Molded part			
	Water wash	MeOH wash	Acetone wash	MeOH wash	Acetone wash		
Montell Pro-Fax SD-242	4,4	3,4,0,4	4,4	0,0,1,0	1,1,0,0	Matrix Basket Material, PP Copolymer	Slight signal obtained with acetone-washed, intact matrix basket and aqueous reaction.; no signal with bound template
Montell Pro-Fax SR-857M	2,1	4,4,3,4	4,4	0,0	0,0	SCIP Material-Yellowish Parts, PP Copolymer	Previous experiments using boiled or untreated reagent tubes were unsuccessful
Montell Pro-Fax PD-702	0,2	3,2,0,1	3,3	0,0	0,0	PCR Tube Material PP Homopolymer	
Huntsman PP-P4G4K-038	4,4	4,4,4,3	4,4	0,0	1,0	Spin Basket Material, PP Homopolymer	Pieces cut from molded basket, washed, dried, added to aqueous reaction in PCR tube
Millbrandt PP	0,1	2,1,0,0	0,0	0,0	0,0	Shim Material. Unknown Extrusion PP Material	Pieces cut from molded part, equivalent weight added to aqueous reaction.
Equistar P - 51S30V	0,1	1,1,0,0	0,0	0,0	0,0	SPE Tube Material, PP Homopolymer	Pieces cut from molded part, equivalent weight added to aqueous reaction.

Table 9. Summary of SDA inhibition studies obtained with various polypropylene (PP) sources and types. The lateral flow test results are assigned numbers from 0 to 4 based on the visual intensity of the blue test line.

INTERIM DEMONSTRATION SUMMARY (OCTOBER 1999 AT XTRANA):

In this demonstration extraction, amplification and detection were achieved in the prototype device. Figure 17 is a photograph of two devices shortly after the rotation of the products of the SDA reaction onto the lateral flow strip laminate.

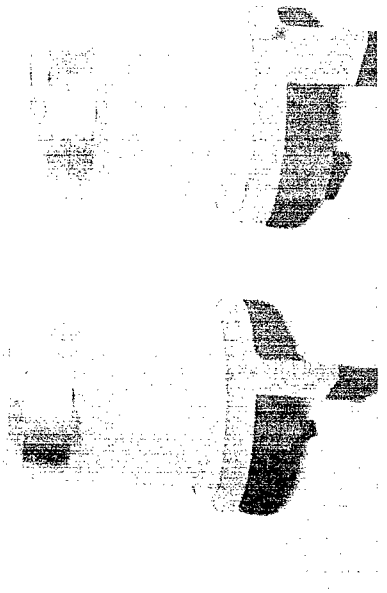


Figure 17. Two SCIP devices after extraction, amplification and detection with 10^7 copies of recombinant plasmid DNA containing *Y. pestis* DNA sequences.

Figure 18 is a summary of results obtained with devices tested during and two days before the interim demonstration. Of the 27 "demo devices" built, all generated the correct result. Moreover, the devices did not malfunction during use. Of the 25 positives run, all generated the correct result. This group consisted of 16 devices for extraction, amplification and detection, 8 for amplification and detection only and one which was checked for detection capability before the evaluation. The two negative controls generated the correct result.

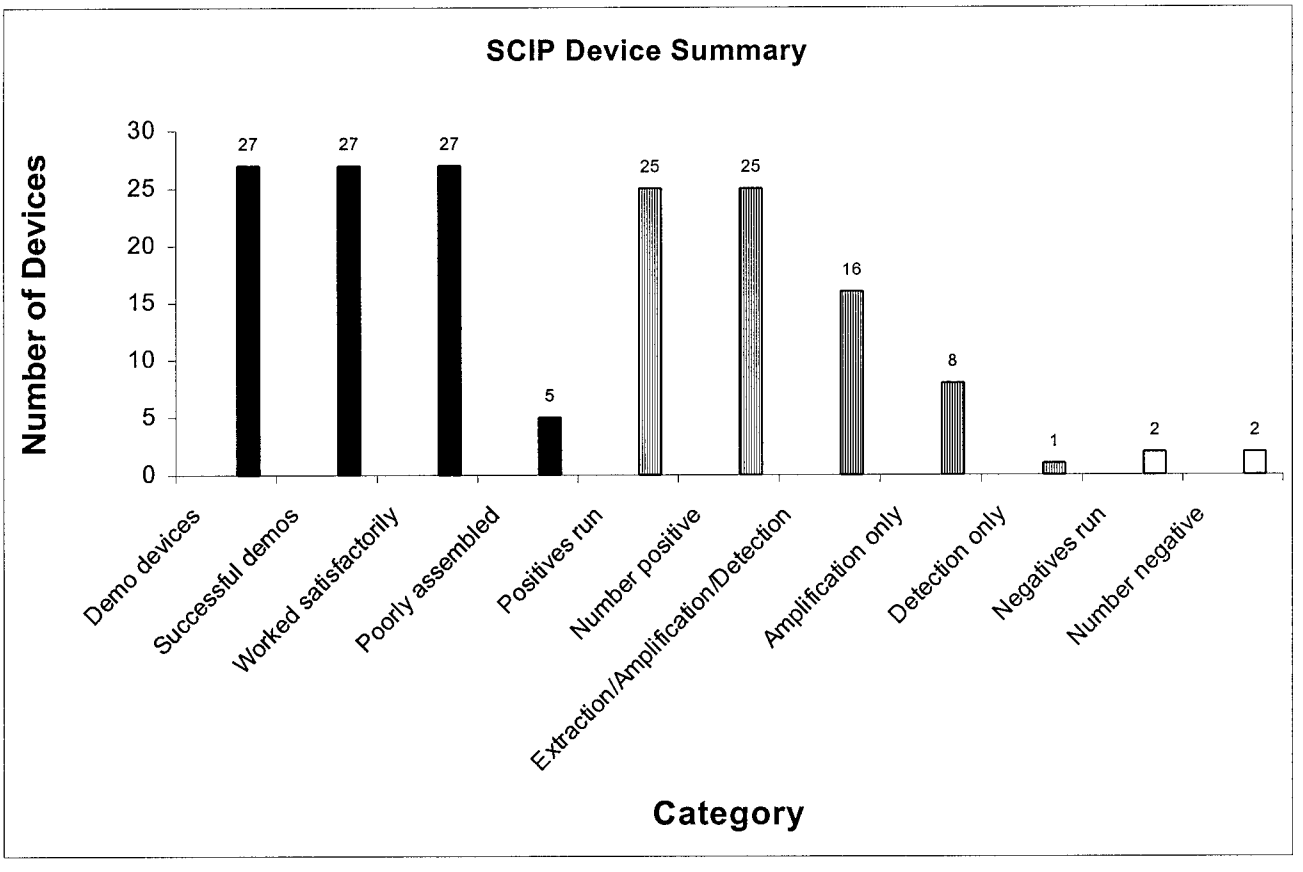


Figure 18. Summary of results from the interim demonstration conducted in October 1999 at Xtrana, Inc.

These results were encouraging and were the first demonstration of the feasibility of the concept of extraction, amplification and detection of a specific nucleic acid target in a disposable device.

FINAL DEMONSTRATION SUMMARY

In order to verify that integrity of the devices and reagents were not compromised following shipment, we conducted a series of evaluations preceding the final demonstration. Tables 10 and 11 reflect the first set of results obtained with the devices.

10/28/00 Saturday AM reagent QC/SCIP evaluation

a.

SCIP extraction amplification *

Copy number	Result	LF Band Intensity
$1x10^6$	-	0
$1x10^5$	-	0
$1x10^4$	-	0

b.

Control PCR tube extraction amplification

Copy number	Result	LF Band Intensity
$1x10^6$	+	4
$1x10^5$	+	4
$1x10^4$	+	4
$1x10^3$	-	0
$1x10^2$	+	4

* Slurry transfer volume was not fully removed

Tables 10a and 10b. First comparison between SCIP and control devices evaluated at USAMRIID. Note that devices initially failed to generate the expected result due to the incomplete removal of buffer from reaction area.

10/29/00 Sunday AM reagent QC/SCIP evaluation

a.

SCIP extraction amplification **

Copy number	Result	LF Band Intensity
$1x10^6$	+	2
$1x10^5$	+	2
$1x10^4$	+	3

b.

Control PCR tube extraction amplification

Copy number	Result	LF Band Intensity
$1x10^6$	+	3
$1x10^5$	+	3
$1x10^4$	+	4
$1x10^3$	+	4
$1x10^2$	+	4
$1x10^5$ XBQC	+	4

** Slurry transfer volume was completely removed

Tables 11a and 11b. Second comparison between SCIP and control devices evaluated at USAMRIID.

10/30/00 Monday AM reagent QC/SCIP evaluation

a.

SCIP extraction amplification **

Copy number	Result	LF Band Intensity
$1x10^6$	+	4
$1x10^5$	+	4
$1x10^4$	+	3
$1x10^3$	+	4

b.

Control PCR tube extraction amplification

Copy number	Result	LF Band Intensity
$1x10^6$	+	4
$1x10^5$	+	4
$1x10^4$	+	4
$1x10^3$	+	4
$1x10^2$	+	4
Negative	+	0

Tables 12a and 12b. Third comparison between SCIP and control devices evaluated at USAMRIID.

10/30/00 Monday PM Demonstration

a.

SCIP extraction amplification

Copy number	Result	LF Band Intensity
$1x10^6$	+	3
$1x10^6$	+	3
$1x10^5$	+	2
$1x10^5$	+	4
$1x10^4$	+	1
$1x10^4$	+	2
$1x10^3$	+	1
$1x10^3$	+	3

b.

Control PCR tube extraction amplification

Copy number	Result	LF Band Intensity
$1x10^5$	+	3
$1x10^4$	+	3
$1x10^3$	+	3
$1x10^2$	+	3
Negative	+	0

Tables 13a and 13b. Forth comparison between SCIP and control devices evaluated at USAMRIID. These represent the actual results obtained during the demonstration on 10-30-00.

The SCIP devices used on the official demonstration performed according to expectations and the target DNA was detectable at the 1 x 10^3 level. The intensity of the signals obtained for the devices were generally weaker than the controls (Tables 12 and 13).

CURRENT STATUS OF DETECTION LIMIT WITH *Y. pestis* GENOMIC DNA

Figure 19 summarizes the limit of detection for the *Y. pestis* target DNA. The results suggest that extraction, amplification and detection performed outside the SCIP device results in a lower detection limit.

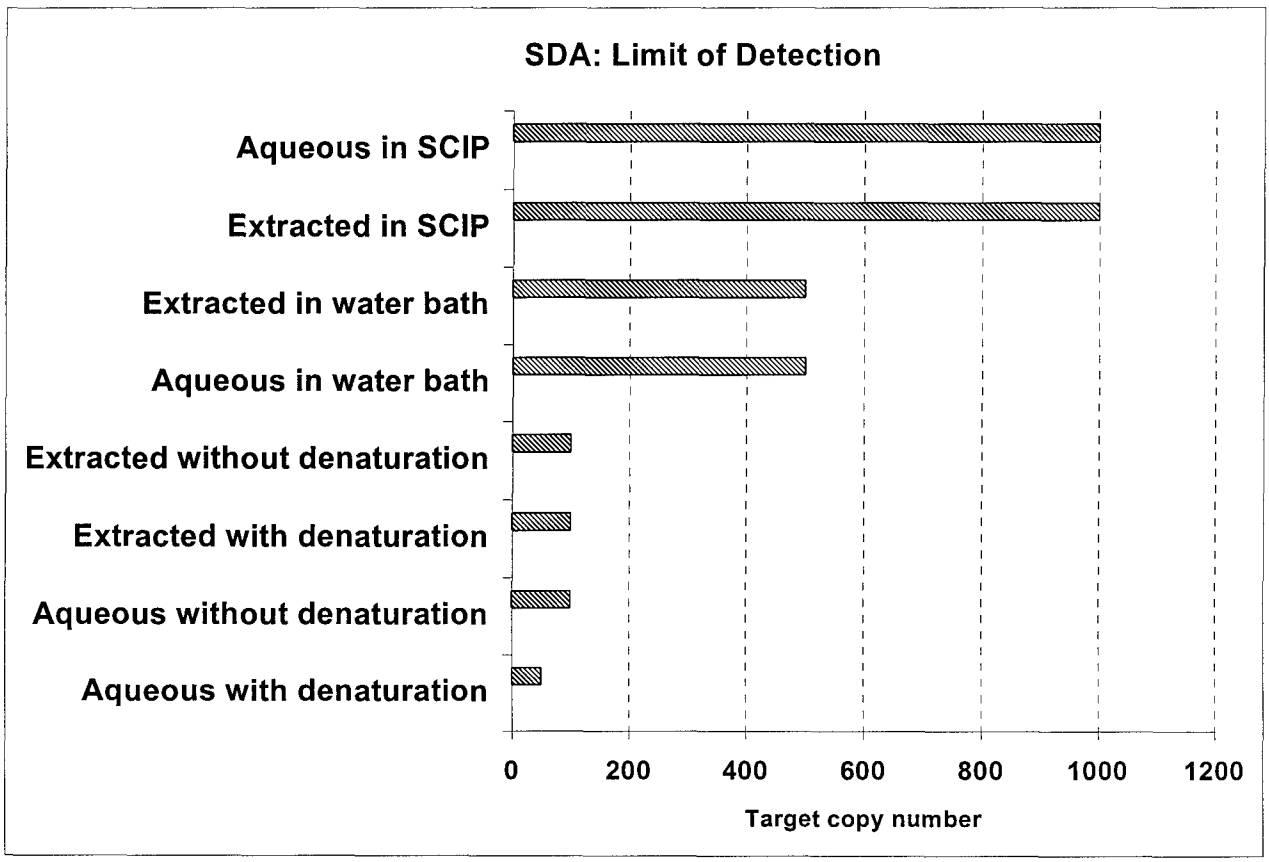


Figure 19. Summary of SDA performance in under the conditions indicated.

COMPARISON BETWEEN THE INTERIM AND FINAL DEMONSTRATIONS

INTERIM DEMONSTRATION	FINAL DEMONSTRATION
Purified rDNA target used (low complexity target)	Total genomic DNA used (high complexity target)
Detection limit = 1 x 10^7 copies	Detection limit = 1 x 10^3 copies
Incomplete LFT laminate	Complete LFT strip laminate with dried conjugates
Target denaturation required	Completely isothermal
Interface of all 3 steps demonstrated in SCIP	SCIP amplification & detection demonstrated

Table 14. Comparison between the interim and final demonstrations for the SCIP devices. The interim demonstration was conducted at Xtrana, Inc. Denver, CO., and the final demonstration was conducted at USAMRIID, Ft. Detrick, MD.

KEY RESEARCH ACCOMPLISHMENTS

SUMMARY OF ACHIEVEMENTS

Extraction

- Used *Y. pestis* genomic DNA
- Conditions optimized with statistical DoE
- Extraction achieved in plasma
- Sample volume: 200 µl to 1 ml
- Eliminated heat denaturation step

Amplification

- All conditions optimized with statistical DoE
- Homogeneous reactions achieved
- Heterogeneous reaction achieved
- Scaled up to 100 µl reactions
- Omitted 95°C denaturation
- Internal positive control developed
- Amplification with SCIP device in water bath
- Sensitivity: 50 copies in solution and 100 copies off Xtra Bind™

Detection

- Lateral flow strips were constructed with dried conjugates in release pads.
- Complete laminates were shown to be stable throughout the incubation phases of the SDA reactions.

REPORTABLE OUTCOMES

This program provided us with a solid understanding of the adaptation of an isothermal amplification technique to a point-of-care device. It shed insight into the design of more robust devices in which fluid management and specific focused heating play an important role. In addition, the program resulted in the development of a reliable and disposable ready-to-use lateral flow detection unit for use in molecular diagnostics.

PATENT

Gerdes, J.C., Jankovsky, L.D., and Kozwich, D.L. 1999. Self-contained device integrating nucleic acid extraction amplification and detection. US5955351: See Appendix VII.

MANUSCRIPTS, ABSTRACTS AND PRESENTATIONS

Mondesire R., Beard S., Gerdes J., Kozwich D., Woronoff S., Xtrana, Inc., Denver, CO. 2000. Nucleic acid extraction, amplification, and visual detection in a contained, closed system. Poster Presentation. 32nd Annual Oak Ridge Conference. Boston, MA. AACC.

Mondesire, R.R., Kozwich, D.L., Johansen, K.A., Gerdes, J.C. and Beard, S.E. 2000. Solid-phase nucleic acid extraction, amplification and detection in molecular diagnostics. May/June Issue. IVD Technology Supplement. 9-13

Woronoff, H., Roehl, C. Landau, K., Roehl, P., Kozwich, D., Gerdes, J., Marmaro, J. and Beard, S. 1999. The Xtra Amp™ Extraction System permits rapid nucleic acid extraction and amplification in a single tube. XII Annual Colorado Biotechnology Symposium. University of Colorado.

CONCLUSIONS

The development of our medical device for the detection of *Y. pestis* present in a human plasma was characterized by three interdependent functions for practical application: 1) extraction of the target nucleic acid from a complex mixture, 2) selective amplification of the target, and 3) detection of the amplified product. Our goal for this phase of the project was to refine and integrate these broad areas in a manner that would allow us to perform these functions in a single device under isothermal reaction conditions. To advance all aspects of the project rapidly, we incorporated a statistical analysis and design of experiment application to all of our experiments. This Design-Expert™ software allowed statistically valid, directed choices to be made with regard to every experimental variable encountered in this project. These functions were demonstrated successfully using the SCIP device in 1999 and 2000.

Feasibility studies performed during the initial phases of the project utilized a recombinant DNA molecule carrying the specific target isolated from *Y. pestis* genomic DNA. We chose a Strand Displacement Amplification (SDA) method to generate detectable product from a low copy source. These experiments demonstrated that existing technology could be used to detect a *Y. pestis* target, albeit at a copy number of 1×10^7 in the SCIP device. In its purified form, this cloned DNA target was of insufficient complexity to assess the practical utility of our system beyond a proof of concept. Instead, we utilized whole genomic DNA that represented a level of genetic sequence complexity that was orders of magnitude higher than had been used previously. The choice of this target molecule imposed greater demands on each component of our integrated device.

Before proceeding to other areas of development, we needed to clearly define the requirements of the amplification reaction that would insure a reproducibly robust reaction. To accomplish this, we evaluated all of the reaction components involved in the generation of amplified product. These optimization studies were successful not only in selecting the most active SDA primer set, but also in defining the optimal reaction buffer and enzyme concentrations that could detect as little as *ten copies* of input target DNA. We observed that this optimization process was primer-specific. Additionally, over time or with incorporation of newly synthesized primers of the same nucleic acid sequence, these idealized conditions tended to drift. The reasons for these subtle changes were unclear, but nevertheless readily apparent. An awareness of this possibility allowed us to rapidly employ the Design-Expert program as needed to quickly restore robust reaction conditions to the system.

Once we had identified the primer set, the salt, co-solvent concentrations and the enzyme specific activities required, we challenged the existing chemistry by amplifying an immobilized DNA target extracted from spiked human plasma. This step required that we address two issues simultaneously. First, we formulated a buffer that enhanced binding of DNA to our solid phase matrix, Xtra Bind™. Although we elected to screen a large number of buffer formulations, use of the statistical design/analysis program facilitated rapid decision-making to result in defining the critical ingredients necessary to promote rapid and stable DNA binding to the matrix. To confirm this activity independently we

monitored nucleic acid binding to this solid phase with fluorescently labeled oligonucleotides. Supported by the experimental data summarized in Appendix III, the binding conditions selected were compatible with efficient capture of the input target DNA. Further, the amount of Xtra Bind™ included in the SCIP device was determined to be such that it would not surpass the binding saturation point even in the presence of a large concentration of input DNA. Second, the amplification conditions we had defined previously needed to retain their activity in the presence of residual protein and nucleic acid contributed by the plasma sample. Following extraction and capture of the *Y. pestis* genomic DNA, the amplification conditions were challenged to generate detectable product from a bound template. Since binding is non-selective, amplification had to proceed in the presence of not only *Y. pestis* genomic DNA, but also in the context of any cellular or plasma components that are retained by the Xtra Bind™ material. Under these rigorous conditions, we were able to detect 5×10^2 copies of *Y. pestis* DNA at a high level of precision and specificity.

The transfer of this technology to the SCIP device proved to be problematic initially. Through aggressive troubleshooting we recognized that the source of the problem was independent of the optimized amplification, extraction, and detection technologies. It became clear that the inhibitory effect on amplification of the captured DNA could be traced to the plastic used to create the molded reagent tube for the SCIP device. This problem was not apparent when the device was used rDNA at high copy number. To circumvent this effect, we developed a modified SCIP device and restored the functional activity we had observed previously in the amplification reaction. Clearly, the modified design does not represent a final rendering of the SCIP device, however, several qualities inherent in this product provide a basis for future generations of the device. Specifically, it was very encouraging that the chemistries capable of providing a detection level that is superior to existing technology were sustained through several design changes of the medical device. That these interdependent activities are sufficiently robust to tolerate alterations in the platform, is a good indication that their broad scope can be exploited further in this setting and in future applications.

APPENDICES

Appendix I Oligonucleotide Designs

A. SDA primer designs

1.8 set: Designed according to BD Biosciences recommendation. Located within the caf1 region of pPMT1 plasmid. [85 bp amplicon length].

S1.8 5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
S2.8 5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
B1.8 5' CGAACTCTGCTCAAC 3'
B2.8 5' TATTCTCGTCGTAGAT 3'
DP1.8 5' ATAGGTGAGAGTAGG 3'
DP2.8 5' ATCATATACCCGTTAG 3'

Description

	Tm °C	% GC	Length (bp)
S upper target	46	44	16
S lower target	44	57	14
B1	44	53	15
B2	44	38	16
DP1	46	47	15
DP2	44	38	16

1.8 Primer Set Design Specification

Upper target

Upper Primer. CAF1M:82657U16
Upper Primer: the most stable 3'-dimer: 2 bp, -1.9 kcal / mol

```
5' GCAAGCAAAGAGTATG 3'
   | |
 3' GTATGAGAAACGAACG 5'
```

Upper Primer: the most stable dimer overall: 2 bp, -3.1 kcal / mol

```
          5' GCAAGCAAAGAGTATG 3'
                 | |
3' GTATGAGAAACGAACG 5'
```

No hairpin stems of at least 3 bp

Upper target Tm

Upper Primer, CAF1M:82657U16

Td = 46.5° [nearest neighbor method]
Tm = 64.4° [%GC method]
Tm = 46° [2(A+T)° + 4(G+C)° method]

Lower target

Lower Primer, CAF1M:82713L14

Lower Primer: no 3'-terminal dimer formation
Lower Primer: the most stable dimer overall: 2 bp, -3.6 kcal / mol

```
      5' TAACGCCAGCAGCA 3'
            | |
   3' ACGACGACCGCAAT 5'
```

No hairpin stems of at least 3 bp

Lower target Tm

Lower Primer, CAF1M:82713L14

Td = 52.2° [nearest neighbor method]
Tm = 65.4° [%GC method]
Tm = 44° [2(A+T)° + 4(G+C)° method]

Upper target vs. Lower target

Primers: CAF1M:82657U16 CAF1M:82713L14

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.9 kcal / mol

```
   5' GCAAGCAAAGAGTATG 3'
                    | |
         3' ACGACGACCGCAAT 5'
```

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.9 kcal / mol

```
   5' GCAAGCAAAGAGTATG 3'
                    | |
               3' ACGACGACCGCAAT 5'
```

Upper/Lower: the most stable dimer overall: 2 bp, -3.1 kcal / mol

```
         5' GCAAGCAAAGAGTATG 3'
               | |
   3' ACGACGACCGCAAT 5'
```

Primer to Primer product info

Optimal Annealing Temperature: 46.6° (Max: 56.0°)

	Position and Length		Tm [°C]	GC [%]	3' ΔG [kcal/mol]
Product	70		79.5	47.1	-----
Upper Primer	82657	16	53.0	43.8	-5.7
Lower Primer	82713	14	60.3	57.1	-8.5

Primer to Primer product

Upper Primer, CAF1M:82657U70

Upper Primer: the most stable 3'-dimer: 2 bp, -1.0 kcal / mol

```
5' GCAAGCAAAGAGTATGGCGTGACTATAGGTGAGAGTAGGATCATATACCCGTTAGATGCTGCTGGCGTTA 3'
                 ||
          3' ATTGCGGTCGTCGTAGATTGCCCATATACTAGGATGAGAGTGGATATCAGTGCGGTATGAGAAACGAACG 5'
```

Upper Primer: the most stable dimer overall: 6 bp, -6.7 kcal / mol

```
                    5' GCAAGCAAAGAGTATGGCGTGACTATAGGTGAGAGTAGGATCATATACCCGTTAGATGCTGCTGGCGTTA 3'
                                                  ||||||
3' ATTGCGGTCGTCGTAGATTGCCCATATACTAGGATGAGAGTGGATATCAGTGCGGTATGAGAAACGAACG 5'
```

Hairpin: ΔG = 0.6 kcal / mol, Loop = 8 nt, Tm = 4°

```
5' GCAAGCAAAGAGTATGGCGTGACTATAGGTGAGAGT-
                                  |||    |
  3' ATTGCGGTCGTCGTAGATTGCCCATATACTAGGA-
```

S1

Upper Primer, untitled:1U40

Upper Primer: the most stable 3'-dimer: 2 bp, -1.9 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
      ||
   3' GTATGAGAAACGAACGGGGCTCTTCAGACCACGACTTAGG 5'
```

Upper Primer: the most stable dimer overall: 2 bp, -3.6 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
                    ||
  3' GTATGAGAAACGAACGGGGCTCTTCAGACCACGACTTAGG 5'
```

Hairpin: ΔG = 0.6 kcal / mol, Loop = 8 nt, Tm = 12°

```
5' GGATTCAGCACCAGACTTCTCG-
                    |||  |
   3' GTATGAGAAACGAACGGG-
```

S2

Lower Primer, untitled:87L38

Lower Primer: the most stable 3'-dimer: 2 bp, -1.9 kcal / mol

```
5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
      ||
   3' ACGACGACCGCAATGGGCTCAGTCAGTAATGTACGACA 5'
```

Lower Primer: the most stable dimer overall: 4 bp, -5.3 kcal / mol

```
                          5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
                                ||||
3' ACGACGACCGCAATGGGCTCAGTCAGTAATGTACGACA 5'
```

Hairpin: ΔG = 0.8 kcal / mol, Loop = 10 nt, Tm = 3°

```
                      5' ACAGCATGT-
                               |||  |
3' ACGACGACCGCAATGGGCTCAGTCAGTAA-
```

S1 VS. S2

Primers: untitled:1U40 untitled:87L38

Upper/Lower: the most stable 3'-dimer: 3 bp, -3.4 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
                                        |||
      3' ACGACGACCGCAATGGGCTCAGTCAGTAATGTACGACA 5'
```

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.9 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
                                       ||
                                3' ACGACGACCGCAATGGGCTCAGTCAGTAATGTACGACA 5'
```

Upper/Lower: the most stable dimer overall: 3 bp, -6.2 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
                  |||  |||      ||||
               3' ACGACGACCGCAATGGGCTCAGTCAGTAATGTACGACA 5'
```

DP1

Upper Primer, CAF1M:82681U15

Upper Primer: no 3'-terminal dimer formation

Upper Primer: the most stable dimer overall: 2 bp, -1.5 kcal / mol

```
          5' ATAGGTGAGAGTAGG 3'
                ||
  3' GGATGAGAGTGGATA 5'
```

No hairpin stems of at least 3 bp

DP1 Tm

Upper Primer, CAF1M:82681U15

Td = 35.5° [nearest neighbor method]
Tm = 63.5° [%GC method]
Tm = 44° [2(A+T)° + 4(G+C)° method]

DP2

Upper Primer, CAF1M:82696U16

Upper Primer: no 3'-terminal dimer formation

Upper Primer: the most stable dimer overall: 4 bp, -4.0 kcal / mol

```
      5' ATCATATACCCGTTAG 3'
           ||||
  3' GATTGCCCATATACTA 5'
```

No hairpin stems of at least 3 bp

DP2 Tm

Upper Primer, CAF1M:82696U16

Td = 42.2° [nearest neighbor method]
Tm = 61.8° [%GC method]
Tm = 44° [2(A+T)° + 4(G+C)° method]

S1 VS. DP1

Primers: untitled:1U40 untitled:126L15

Upper/Lower: no 3'-terminal dimer formation

Upper/Lower: the most stable 3'-dimer: 2 bp, -3.1 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
             ||      |||
        3' GGATGAGAGTGGATA 5'
```

Upper/Lower: the most stable dimer overall: 4 bp, -6.3 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
           ||||
3' GGATGAGAGTGGATA 5'
```

S2 VS. DP1

Primers: untitled:45U38 untitled:126L15

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.9 kcal / mol

```
5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
                                  ||
                          3' GGATGAGAGTGGATA 5'
```

Upper/Lower: the most stable 3'-dimer: 2 bp, -3.1 kcal / mol

```
5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
                               ||
                        3' GGATGAGAGTGGATA 5'
```

Upper/Lower: the most stable dimer overall: 4 bp, -4.5 kcal / mol

```
5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
              ||||
        3' GGATGAGAGTGGATA 5'
```

DP2 VS. DP1

Primers: untitled:106U16 untitled:126L15

Upper/Lower: no 3'-terminal dimer formation

Upper/Lower: the most stable 3'-dimer: 2 bp, -3.1 kcal / mol

```
5' ATCATATACCCGTTAG 3'
        ||
   3' GGATGAGAGTGGATA 5'
```

Upper/Lower: the most stable dimer overall: 3 bp, -4.4 kcal / mol

```
  5' ATCATATACCCGTTAG 3'
        |||
3' GGATGAGAGTGGATA 5'
```

S1 VS. DP2

Primers: untitled:1U40 untitled:126L16

Upper/Lower: the most stable 3'-dimer: 4 bp, -4.4 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
                                    ||||
                              3' GATTGCCCATATACTA 5'
```

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.6 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
                  ||
            3' GATTGCCCATATACTA 5'
```

Upper/Lower: the most stable dimer overall: 4 bp, -9.8 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
                      ||||
                3' GATTGCCCATATACTA 5'
```

S2 VS. DP2

Primers: untitled:45U38 untitled:126L16

Upper/Lower: no 3'-terminal dimer formation

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.6 kcal / mol

```
5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
                  ||
            3' GATTGCCCATATACTA 5'
```

Upper/Lower: the most stable dimer overall: 6 bp, -12.1 kcal / mol

```
5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
                      ||||||
                3' GATTGCCCATATACTA 5'
```

B1

Upper Primer, CAF1M:82628U15

Upper Primer: no 3'-terminal dimer formation

Upper Primer: the most stable dimer overall: 2 bp, -3.6 kcal / mol

```
          5' CGAACTCTGCTCAAC 3'
             ||
3' CAACTCGTCTCAAGC 5'
```

No hairpin stems of at least 3 bp

B1 Tm

Upper Primer, CAF1M:82628U15

Td = 44.8° [nearest neighbor method]
Tm = 66.2° [%GC method]
Tm = 46° [2(A+T)° + 4(G+C)° method]

B2

Lower Primer, CAF1M:82777L16

Lower Primer: the most stable 3'-dimer: 2 bp, -1.5 kcal / mol

```
5' TATTCTCGTCGTAGAT 3'
   ||
 3' TAGATGCTGCTCTTAT 5'
```

Lower Primer: the most stable dimer overall: 2 bp, -3.6 kcal / mol

```
   5' TATTCTCGTCGTAGAT 3'
      ||
3' TAGATGCTGCTCTTAT 5'
```

Hairpin: ΔG = 1.1 kcal / mol, Loop = 6 nt

```
5' TATTCTCGT
   |||
 3' TAGATGC
```

B2 T_m

Lower Primer, CAF1M:82777L16

Td = 41.6° [nearest neighbor method]
Tm = 61.8° [%GC method]
Tm = 44° [2(A+T)° + 4(G+C)° method]

S1 VS. B1

Primers: untitled:1U40 untitled:165L15

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.9 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
                                          ||
                                3' CAACTCGTCTCAAGC 5'
```

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.3 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
                                        ||
                              3' CAACTCGTCTCAAGC 5'
```

Upper/Lower: the most stable dimer overall: 4 bp, -6.6 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
    |  ||||      |
  3' CAACTCGTCTCAAGC 5'
```

S2 VS. B1

Primers: untitled:45U38 untitled:165L15

Upper/Lower: the most stable 3'-dimer: 4 bp, -6.6 kcal / mol

```
5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
                                  ||||
                          3' CAACTCGTCTCAAGC 5'
```

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.3 kcal / mol

```
5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
       ||      |     |||
     3' CAACTCGTCTCAAGC 5'
```

Upper/Lower: the most stable dimer overall: 5 bp, -8.2 kcal / mol

```
5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
                                |||||
                          3' CAACTCGTCTCAAGC 5'
```

DP1 VS. B1

Primers: untitled:87U15 untitled:165L15

Upper/Lower: no 3'-terminal dimer formation

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.3 kcal / mol

```
5' ATAGGTGAGAGTAGG 3'
     ||      |
   3' CAACTCGTCTCAAGC 5'
```

Upper/Lower: the most stable dimer overall: 5 bp, -6.1 kcal / mol

```
5' ATAGGTGAGAGTAGG 3'
   |   |  |||||   |
3' CAACTCGTCTCAAGC 5'
```

DP2 VS. B1

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.6 kcal / mol

```
5' ATCATATACCCGTTAG 3'
      |  |||
   3' CAACTCGTCTCAAGC 5'
```

Upper/Lower: the most stable 3'-dimer: 3 bp, -3.2 kcal / mol

```
5' ATCATATACCCGTTAG 3'
          |||
       3' CAACTCGTCTCAAGC 5'
```

Upper/Lower: the most stable dimer overall: 2 bp, -3.6 kcal / mol

```
    5' ATCATATACCCGTTAG 3'
                 ||
3' CAACTCGTCTCAAGC 5'
```

B2 VS B1

Primers: untitled:126U15 untitled:165L15

Upper/Lower: no 3'-terminal dimer formation

Upper/Lower: no 3'-terminal dimer formation

Upper/Lower: the most stable dimer overall: 2 bp, -3.6 kcal / mol

```
              5' CGAACTCTGCTCAAC 3'
                 ||
3' CAACTCGTCTCAAGC 5'
```

S1 VS. B2

Primers: untitled:1U40 untitled:165L15

Upper/Lower: no 3'-terminal dimer formation

Upper/Lower: the most stable 3'-dimer: 3 bp, -3.2 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
                    |||
                 3' AGATGCTGCTCTTAT 5'
```

Upper/Lower: the most stable dimer overall: 2 bp, -3.6 kcal / mol

```
5' GGATTCAGCACCAGACTTCTCGGGGCAAGCAAAGAGTATG 3'
                  ||
               3' AGATGCTGCTCTTAT 5'
```

S2 VS B2

Primers: untitled:45U38 untitled:165L15

Upper/Lower: no 3'-terminal dimer formation

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.6 kcal / mol

```
5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
                    ||
                 3' AGATGCTGCTCTTAT 5'
```

Upper/Lower: the most stable dimer overall: 3 bp, -4.9 kcal / mol

```
5' ACAGCATGTAATGACTGACTCGGGTAACGCCAGCAGCA 3'
                      |||
                   3' AGATGCTGCTCTTAT 5'
```

DP1 VS. B2

Primers: untitled:87U15 untitled:165L15

Upper/Lower: no 3'-terminal dimer formation

Upper/Lower: no 3'-terminal dimer formation

Upper/Lower: the most stable dimer overall: 4 bp, -4.8 kcal / mol

```
    5' ATAGGTGAGAGTAGG 3'
       |  ||||  |
 3' AGATGCTGCTCTTAT 5'
```

DP2 VS. B2

Primers: untitled:106U16 untitled:165L15

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.6 kcal / mol

```
  5' ATCATATACCCGTTAG 3'
        |  ||
     3' AGATGCTGCTCTTAT 5'
```

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.6 kcal / mol

```
  5' ATCATATACCCGTTAG 3'
     ||  |  |  |
   3' AGATGCTGCTCTTAT 5'
```

Upper/Lower: the most stable dimer overall: 2 bp, -3.6 kcal / mol

```
  5' ATCATATACCCGTTAG 3'
        |  ||
     3' AGATGCTGCTCTTAT 5'
```

B1 VS. B2

Primers: untitled:126U15 untitled:165L15

Upper/Lower: the most stable 3'-dimer: 2 bp, -1.3 kcal / mol

```
  5' CGAACTCTGCTCAAC 3'
        |  |  ||
     3' AGATGCTGCTCTTAT 5'
```

Upper/Lower: the most stable 3'-dimer: 3 bp, -3.2 kcal / mol

```
  5' CGAACTCTGCTCAAC 3'
      |||  |
   3' AGATGCTGCTCTTAT 5'
```

Upper/Lower: the most stable dimer overall: 3 bp, -5.2 kcal / mol

```
      5' CGAACTCTGCTCAAC 3'
         |||
3' AGATGCTGCTCTTAT 5'
```

Y. pestis target sequence

5'GCAAGCAAAGAGTATGGCGTGACTATAGGTGAGAGTAGGATCATATA
CCCGTTAGATGCTGCTGGCGTTA 3'

Upper Primer, CAF1M:82657U70

Upper Primer: the most stable 3'-dimer: 2 bp, -1.0 kcal / mol

```
  5' GCAAGCAAAGAGTATGGCGTGACTATAGGTGAGAGTAGGATCATATACCCGTTAGATGCTGCTGGCGTTA 3'
           |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
        3' ATTGCGGTCGTCGTAGATTGCCCATATACTAGGATGAGAGTGGATATCAGTGCGGTATGAGAAACGAACG 5'
```

Upper Primer: the most stable dimer overall: 6 bp, -6.7 kcal / mol

```
                    5' GCAAGCAAAGAGTATGGCGTGACTATAGGTGAGAGTAGGATCATATACCCGTTAGATGCTGCTGGCGTTA 3'
                       |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
  3' ATTGCGGTCGTCGTAGATTGCCCATATACTAGGATGAGAGTGGATATCAGTGCGGTATGAGAAACGAACG 5'
```

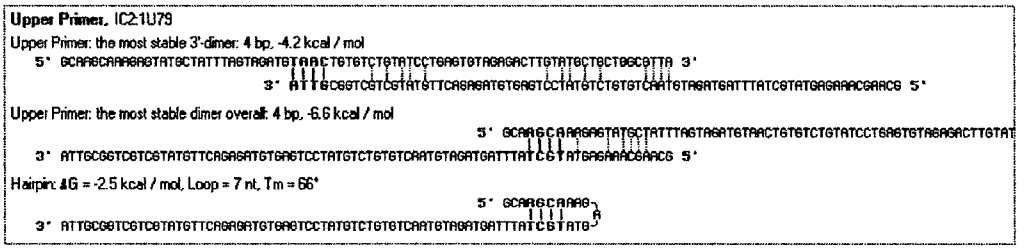
Hairpin: ΔG = 0.6 kcal / mol, Loop = 8 nt, Tm = 4°

```
  5' GCAAGCAAAGAGTATGGCGTGACTATAGGTGAGAGT
     |  |  |  |  |  |  |  |  |  |  |  |  |
    3' ATTGCGGTCGTCGTAGATTGCCCATATACTAGGA
```

Internal Competitive Control

Internal control sequence

5'GCAAGCAAAGAGTATGCTATTTAGTAGATGTAACTGTGTCTGTATCCTG
AGTGTAGAGACTTGTATGCTGCTGGCGTTA 3'



Y. pestis target sequence composition

Upper Primer, CAF1M:82657U70

Tm = 89.9° [%GC method]

Tm (RNA) [1M Na] = 100.0° [%GC method]
Tm (DNA:RNA) [1M Na] = 93.7° [%GC method]

A260/A280 = 1.95 [single strand]
Mr = 21.9 K [one strand]
Mr = 43.3 K [two strands]
µg/OD = 47.4 [dsDNA]

Base	Number and %	
A	20	[28.6%]
C	11	[15.7%]
G	22	[31.4%]
T	17	[24.3%]
A + T	37	[52.9%]
G + C	33	[47.1%]

DNA Melting Temperature in Various Salt and Formamide Concentrations [°C]

[mM]	xSSC	0%	10%	50%
1	(0.006)	43.9	37.4	11.4
10	(0.06)	60.4	53.9	27.9
50	(0.3)	71.8	65.3	39.3
165	(1)	79.9	73.4	47.4
330	(2)	84.2	77.7	51.7
500	(3)	86.5	80.0	54.0
1000	(6)	89.9	83.4	57.4

Internal control sequence composition

Upper Primer, IC2:1U79

Tm = 88.5° [%GC method]

Tm (RNA) [1M Na] = 97.1° [%GC method]
Tm (DNA:RNA) [1M Na] = 90.3° [%GC method]

A260/A280 = 1.89 [single strand]
Mr = 24.6 K [one strand]
Mr = 48.8 K [two strands]
µg/OD = 47.8 [dsDNA]

Base	Number and %	
A	20	[25.3%]
C	11	[13.9%]
G	22	[27.8%]
T	26	[32.9%]
A + T	46	[58.2%]
G + C	33	[41.8%]

DNA Melting Temperature in Various Salt and Formamide Concentrations [°C]

[mM]	xSSC	0%	10%	50%
1	(0.006)	42.5	36.0	10.0
10	(0.06)	59.0	52.5	26.5
50	(0.3)	70.4	63.9	37.9
165	(1)	78.5	72.0	46.0
330	(2)	82.8	76.3	50.3
500	(3)	85.1	78.6	52.6
1000	(6)	88.5	82.0	56.0

Appendix II — Typical SDA experimental set up

***Y.pestis* SDA** Exp Name: 071900E

Purpose: Booster Amplification evaluation. Round 2 Sample #: 30

Primer Sub Mix:

REAGENT:	Stock:	LOT #	1x conc	units	1X Volume	Total to Use
Water					4.98	149.40
S1.8 primer	25	061500 BSI	0.5	µM	1.00	30.00
S2.8primer	25	061500 BSI	0.5	µM	1.00	30.00
B1.8primer	2.5	060800 IDT	0.05	µM	1.00	30.00
B2.8 primer	2.5	061300 IDT	0.05	µM	1.00	30.00
BSA in 5% glycerol	5	081799-04	0.1	µg/µl	1.00	30.00
hDNA stock per µl	355	1200999-06	710	ng	2.00	60.00
dntp's (1mM A,T,G, 7mM C)	10	71100	1	X	5.00	150.00
K_2HPO4	493.1	121799-03	50	mM	5.07	152.10
DMSO	100	062300-12	5	%	2.50	75.00
Glycerol(9.105% Final/rxn)	55	030800-05	6	%	5.45	163.64
			Total Volume:		**30.00**	**900**

Enzyme Sub Mix 1:

REAGENT:	Stock:	LOT #	1x conc	units	1X Volume	Total to Use
Water					5.6	168.00
MgOAC2	165	71100	5	mM	1.52	45.45
Glycerol	55	030800-05	2.445	%	2.22	66.68
BsoB1	160	12-122297 5-00	80	units	0.50	15.00
BstPol	120	15A/110697 4-00	18.9	units	0.16	4.73
			Total Volume:		**10.00**	**300**

Sample Order

1 $1x10^3$ copies 10µl neat
2 $1x10^3$ copies
3 $1x10^2$ copies
4 $1x10^2$ copies
5 $1x10^1$ copies
6 $1x10^1$ copies
7 Negative control
8 $1x10^3$ copies 10µl 1:10 diln
9 $1x10^3$ copies
10 $1x10^2$ copies
11 $1x10^2$ copies
12 $1x10^1$ copies
13 $1x10^1$ copies
14 Negative control

15 $1x10^3$ copies 10µl neat
16 $1x10^3$ copies
17 $1x10^2$ copies
18 $1x10^2$ copies
19 $1x10^1$ copies
20 $1x10^1$ copies
21 Negative control
22 $1x10^3$ copies 10µl 1:10 diln
23 $1x10^3$ copies
24 $1x10^2$ copies
25 $1x10^2$ copies
26 $1x10^1$ copies
27 $1x10^1$ copies
28 Negative control

5 minute Amp product

10 minute Amp product

Appendix III Analysis of Xtra Bind™

Aluminum Oxide Analysis and DNA binding

Introduction:

In the SCIP, Xtrana is applying aluminum oxide as a proprietary and unique material for binding DNA and allowing subsequent on-surface amplification. While silica and other materials have been used in similar applications, they do not allow on-surface application. Aluminum oxide therefore provides the advantages of simpler processing and repeated sample access and archiving. Given the novel and important role of aluminum oxide in SCIP, we performed select analyses to characterize the material and its performance. The Al_2O_3 crystals studied were of the fused and calcined varieties, and were obtained from Aldrich and Washington Mills.

Fourier transform infrared (FTIR) spectroscopy is a powerful analytical tool for characterizing and identifying organic molecules. The IR spectrum of the various forms of Al_2O_3 provided specific information about chemical bonding and molecular structure of this material. We were thus able to select specific forms of this compound that had the relevant features for our application. X-ray diffraction (XRD) is an analytical tool where at X-rays scattering from crystalline materials produces a unique x-ray "fingerprint" of X-ray intensity versus scattering angle that is characteristic of it's crystalline atomic structure. Qualitative analysis is achieved by comparing the XRD pattern of an unknown material to known patterns.

Analytical Methods:

X-ray Diffraction (XRD) Analysis

The samples were analyzed in a Rigaku XRD powder diffraction instrument. The x-ray source was a copper K_α target with a current of 15 mA and a potential of 40 kV. The diffracted x-rays were collected with a scintillation counter and the signal intensity was recorded. The scans were done between 10 and 100 degrees 2θ for most samples at a scan rate of 6 degrees per minute and a step width of 0.1 degrees 2θ. Some selected samples were only scanned from 10 to 80 degrees 2θ.

The samples were prepared by grinding them in an agate mortar and pestle until the particles were 200 mesh or smaller. The ground powder was placed in an aluminum sample holder that is 3.5 cm by 5 cm and has a 2 x 2 cm window for the sample. A glass slide was taped to one side of the window to form a sample well with a non-crystalline bottom. The ground sample was placed in the sample well and the powder smoothed to obtain a flat surface at the top of the well. For small samples the well was filled with modeling clay and a flat layer of the sample formed on the surface of the clay. The whole disk samples were mounted on the clay surface to and pressed into the clay with a flat piece of glass to provide a surface with all the disks in the same plane.

An XRD spectrum of fused, alpha aluminum oxide (Aldrich) is shown in Figure 1a. This aluminum oxide material is the most commonly used alumina in Xtrana's DNA binding and amplification, and shows distinct narrow peaks of significant amplitude. In contrast, an XRD spectrum from a sample of gamma alumina oxide (Alfa AESAR) is shown in Figure 1b. The broader, lower amplitude pattern is characteristic of gamma aluminum oxide. Gamma, aluminum oxide does not work well for binding and amplifying DNA.

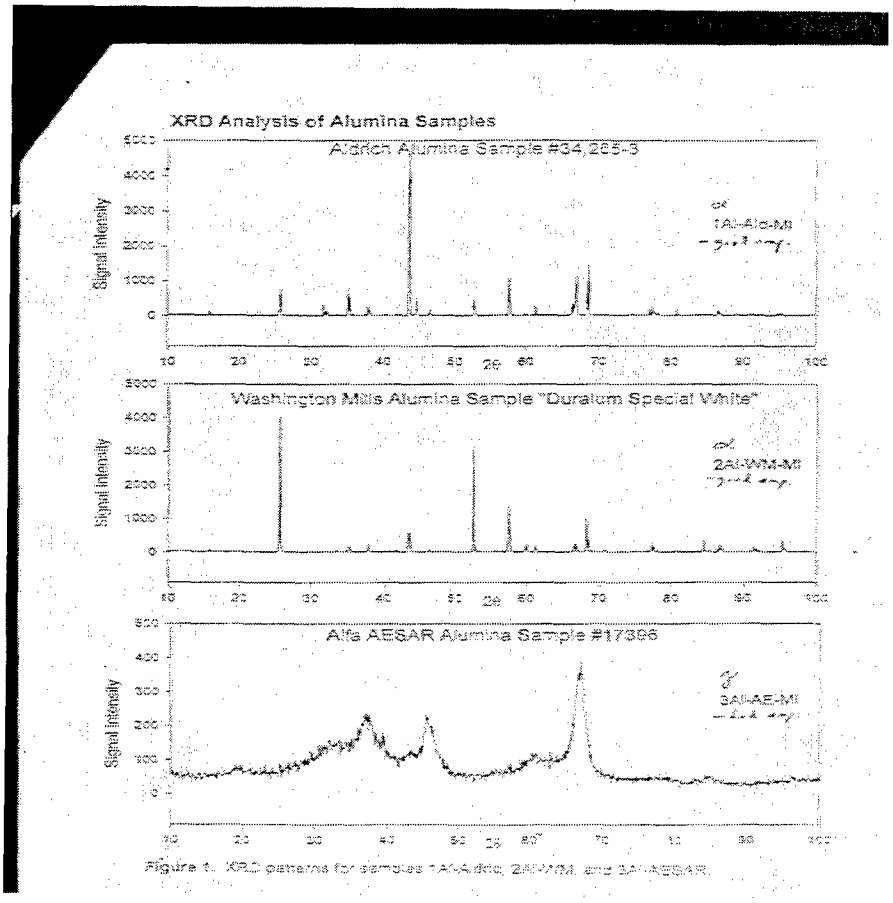


Figure 20. Comparison of XRD spectra of α-Al_2O_3 and γ-Al_2O_3

FTIR Analysis

The samples were analyzed by a Perkin-Elmer 1000 PC with a TGS detector. The scans were taken from 4000 to 450 cm^{-1} in the transmission mode. The samples were ground to a 200 mesh powder in an agate mortar and pestle. About 10-30 mg of sample were mixed with 100-300 mg of KBr powder, pressed at 20,000 psi into a KBr window, and analyzed.

FTIR scans of two different fused, alpha alumina samples and a gamma alumina sample are shown in Figure 2. The spectra are clearly different, and the spectral differences are generally due to the hydration of gamma materials versus the dehydrated state of alpha alumina.

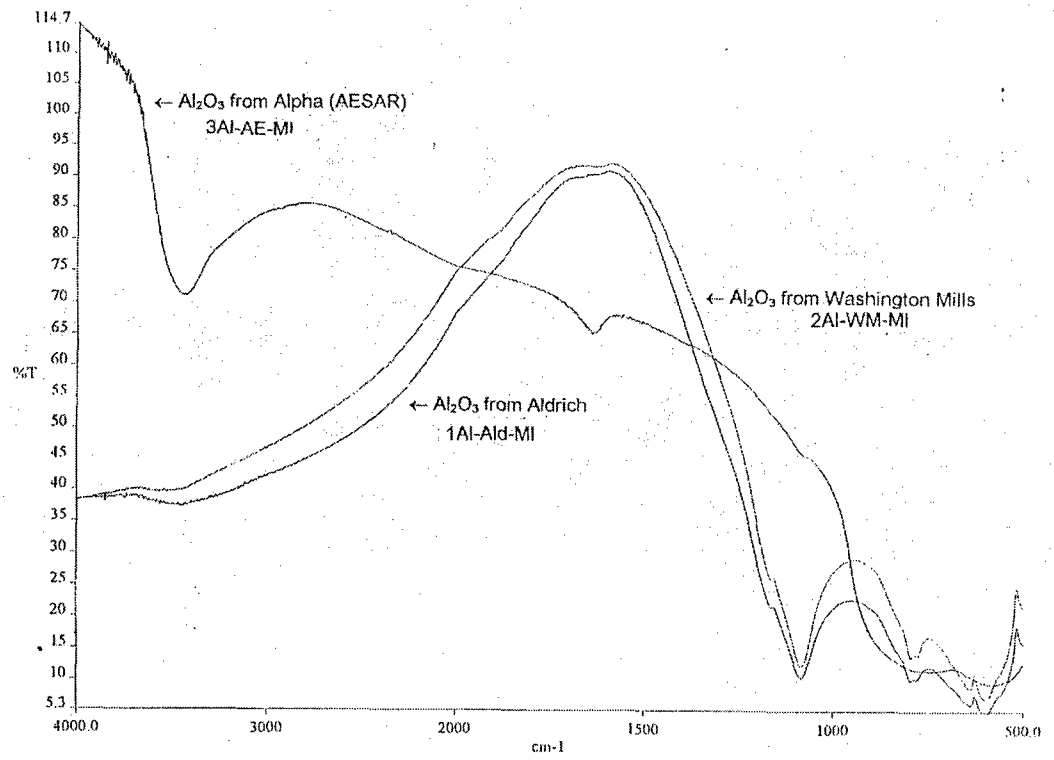


Figure 21. FTIR Spectra from different aluminum oxide samples. The Aldrich and Washington Mills samples are fused, alpha alumina, and the Alpha (AESAR) sample is gamma alumina.

Fluorescence

Direct detection of DNA binding to aluminum oxide was tested using fluorescently labeled oligonucleotides. Oligonucleotides, 18 bases long and labeled at the 5' end with fluorescein, were mixed in solutions from 10 nM to 2.5 μM. PCR buffer was the most common solvent, but deionized water and phosphate buffers were also tried. Oligonucleotide solutions were mixed with measured amounts of aluminum oxide, stored for 15 minutes at room temperature, followed by three or more washes with wash buffer. 50 μl solutions containing aluminum oxide slurry and wash buffer were transferred to

individual wells of 96 well plates and scanned in a Perkin Elmer LS 50B fluorometer. Specific wells also contained appropriate controls.

Figure 22 summarizes the different binding properties of varied types of aluminum oxide. These results indicate that, while fused, alpha aluminum oxide is the most successful aluminum oxide for our application of binding DNA while still allowing enzymatic amplification, this is not because it is the only aluminum oxide that binds DNA. In fact, other types of aluminum oxide appear to have a higher affinity for DNA. Presumably, the lower affinity of fused, alpha aluminum oxide is related to its successful use in amplification, and further study will clarify this important mechanism.

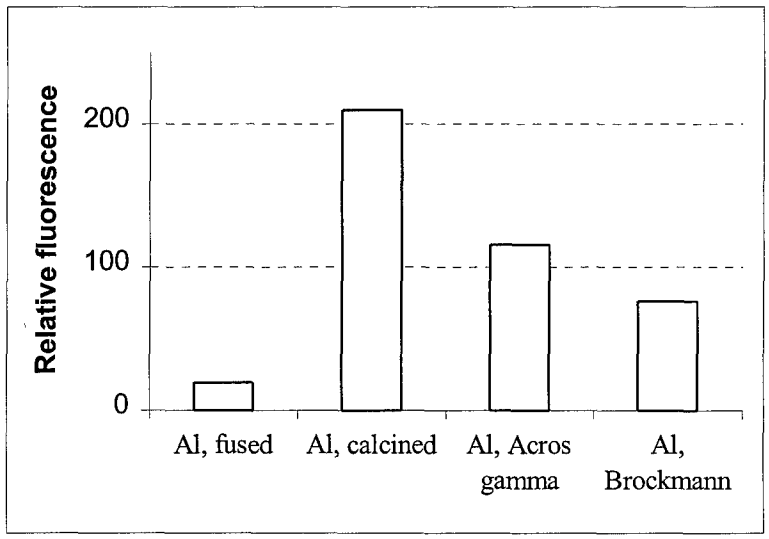


Figure 22. Summary of the different binding properties of varied types of aluminum oxide.

Other experiments with fluorescently labeled DNA investigated DNA binding during flow conditions that are similar to extraction in the SCIP device. One ml solutions of fluorescein-labeled oligonucleotides in lysis buffer flowed through packed beds of different amounts of aluminum oxide. The containers were the SCIP reaction tubes. Results are shown in Figure 2, and indicate that increasing the amount of aluminum oxide can increase the amount of bound DNA until a limit of approximately 40 mg is used.

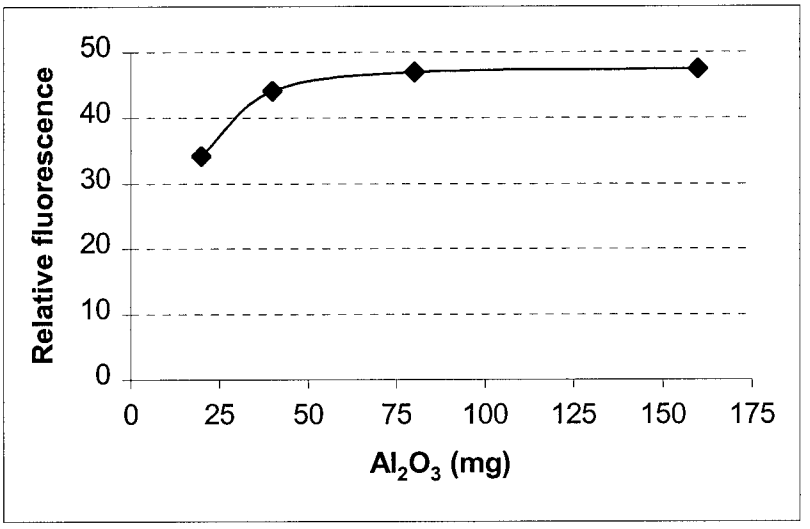


Figure 23. Fluorescence measurements of DNA binding on different amounts of aluminum oxide following unit gravity flow through experiments.

Summary:

Based on these and other experiments with fluor-labeled oligonucleotides, the following observations can be noted:

1. DNA binding to fused, alpha aluminum oxide involves lower affinity than other types of aluminum oxide.
2. Flow through measurements of DNA binding demonstrate binding, and the bound signal depends on the amount of aluminum oxide and the number of DNA molecules in solution.
3. The configuration of the flow chamber (narrow vs. wide) and the concentration of DNA have less significant affects on the signal.
4. The binding capacity of 20 mg of aluminum oxide is in large excess compared to DNA copy numbers of 10^6 and lower.
5. Binding is strong in deionized water and lysis buffer, but is attenuated in proportion to increasing phosphate concentration.

Appendix IV **Components of the SCIP Device**

Part # RD1015
Outer tube: Clear polypropylene

Part # RD1016
Reaction tube: Clear polypropylene

Part # RD1017
Waste tube: Clear polypropylene

Part # RD Shim-01
Shim: polypropylene 0.040" thick

Part # RD1024
Cap for reaction tube cap & handle

Part # RD1028
Thermal transfer-seal disk: Aluminum alloy lubricated with Dupont's KRYTOX®

RD1030
Retainer: Screen disk: Stainless steel

Part # RD: LF-01
Lateral flow strip
Sample pad: Ahlstrom 237
Conjugate pad: Glass fiber #9254 (Lydall Technical Papers) or AccuFlow™ P (Schleicher & Schuell)
Mylar adhesive: 0.5" Adhesives Research;
Nitrocellulose: Hi-Flow™ (Millipore) coated with anti-FITC IgG (lot # 072899)
Absorbent pad: # 470 (3/4" Schleicher & Schuell)
Adhesive backing: G&L Precision Die Cutting (0.01" White PS with GL 187 adhesive)
Note: The sample pad must project 1/16" beyond the strip backing.

Part WA-01
Waste absorbent: (Whatman Grade D28 or equivalent glass fiber/cellulose mixture).

Appendix V: **Collaborators**

Xtrana acknowledges the following participants in this program:

1. Ansys Diagnostics, Lake Forest, CA
2. BD Biosciences, Sparks, MD
3. Advantage Diagnostics, Mountainview CA
4. USAMRIID, Diagnostic Systems Division, Ft. Detrick, MD
5. Dr. Scott Cowley, Colorado School of Mines, Golden, CO.
6. Dr. David Granger, Colorado State University, Ft. Collins, CO.

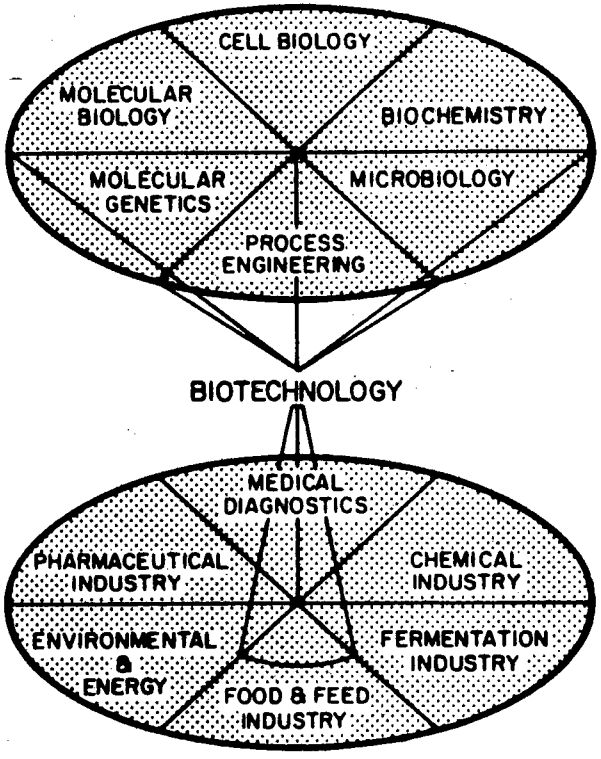
Other Consultants

1. Bob Case & Associates/Product Design Center
2. Stoesser Industries, Mountainview CA.
3. Advantage Diagnostics, Mountainview CA

Appendix VI Reprints of manuscripts

Registration Information
Final Program
Book of Abstracts

TWELFTH ANNUAL COLORADO BIOTECHNOLOGY SYMPOSIUM



14 September 1999
University Memorial Center
University of Colorado
Boulder, Colorado

Sponsored by the
Colorado Institute for
Research in Biotechnology

We gratefully acknowledge Amgen for providing GCSF and financial support. Additional financial support was provided by an advanced graduate student research fellowship from the PhRMA Foundation for LSJ.

Primary author is a graduate student.

Poster 55 – **The Xtra Amp™ Extraction System Permits Rapid Nucleic Acid Extraction and Amplification in a Single Tube,** *Hyun (Sam) Woronoff, Christopher Roehl, Keli Landau, Patrick Roehl, Diane Kozwich, John Gerdes, Jeffrey Marmaro, and Shannon E. Beard, Molecular Innovations, Inc.*

Molecular Innovations, Inc. has developed a non-silica based nucleic acid extraction method, the Xtra Amp™ Extraction System. This simple and rapid system allows for combining nucleic acid extraction and amplification, via either PCR or an isothermal amplification in the same tube. Lysis buffer and sample are added to the Xtra Amp™ tubes, mixed and incubated for 10 minutes. The lysate is removed, the tubes are washed 3 times, and the Xtra Amp™ tubes are ready for amplification master mix addition. This extraction system eliminates transfer and dilution steps resulting from the elution step associated with other extraction methods thus minimizes total handling. Use of a single tube for nucleic acid extraction and amplification makes this an ideal system for sample tracking by barcoding and automated sample processing. The Xtra Amp™ Extraction System does not require vacuum filtration or centrifugation and does not contain carcinogenic or hazardous chemicals. An entire set of 96 samples can be completely extracted with an automated robotic system in less than 15 minutes or manually with a multichannel pipettor in less than 30 minutes. In addition, this system is capable of archiving the extracted nucleic acid for extended periods of time and sequential amplification of the same extracted tube. This novel system has application in all areas of nucleic acid analysis.

Conclusions:

- The Xtra Amp™ Extraction System provides nucleic acid extraction in minutes, and is fully amenable to automation
- Amplification (PCR or isothermal) can occur in the same tube used for extraction
- Multiple targets can be amplified & detected with 1 extraction/1 tube
- DNA can be archived directly in extraction tube for long term storage
- Capture system provides for low copy or higher specificity target isolation

Poster 56 – **Histone-DNA Interactions are Sufficient to Position Nucleosomes on the *PHO5* Gene of *Saccharomyces cerevisiae,*** *John Pilon, Paul, Craig Martens, and Paul Layboum, Colorado State University*

The *PHO5*gene of *Saccharomyces cerevisiae* is of fundamental importance in research into the mechanisms of transcription in the context of chromatin. The gene product encodes a secreted acid phosphatase that is activated in conditions of low phosphate. The repressed state of this gene has four precisely positioned nucleosomes that cover the minimal promoter thereby blocking access of the general transcription machinery to the TATA box. Moreover, the repressed state chromatin structure has a hypersensitive region containing a binding site for the transcriptional activator Pho4p located between two of the positioned nucleosides. The question of how this nucleosomal array, which is essential for proper gene regulation, is created remained unknown. This study has shown that Histone-DNA interactions are sufficient to position nucleosomes and form the observed chromatin structure. Furthermore, we have demonstrated that elastic iosotropic properties of the underlying DNA sequence is strongly implicated in this example of nucleosome positioning. This phenomena is modeled by an algorithm developed by Sivolob et. al. (*Journal of Molecular Biology 1995 April 14;247(5) 918-31*) which uses experimental persistence length values for dinucleotide steps to determine the flexibility of a DNA sequence. A nucleosome is then formed on DNA sequences that require a minimum amount of energy to bend the DNA polymer into a nucleosome.

Primary author is a graduate student.

Solid-phase nucleic acid extraction, amplification, and detection

ROY R. MONDESIRE, DIANE L. KOZWICH, KRISTINE A. JOHANSEN, JOHN C. GERDES, AND SHANNON E. BEARD

The use of molecular technologies for clinical diagnostics doesn't have to be synonymous with high-cost, high-complexity instrumented systems.

Since the introduction of the first FDA-approved clinical laboratory test kit for *Legionella*, by Gen-Probe Inc. (San Diego), molecular diagnostic methods have played an important role in the diagnosis of a variety of diseases.[1,2] Although the market is dominated by infectious-disease diagnostics, molecular tests for cancer and genetic diseases are on the increase. The primary factors that have contributed to the rapid expansion of molecular diagnostic pathology include improvements in nucleic acid extraction techniques, the development of more-efficient DNA-sequencing methods in conjunction with the Human Genome Project, the growing diversity of nucleic acid amplification techniques, the accessibility of several commercially available molecular detection methods, and the introduction of semiautomated instrumentation for probe testing.

In an effort to secure their share of the emerging nucleic acid diagnostics market, several companies are competing to develop new medical devices. Many such companies are developing products based on the relatively complex and instrumented technologies of DNA microarrays and related microfluidics.[3–5] By contrast, our company is focusing on the development of relatively simple solid-phase extraction, isothermal amplification, and rapid detection by lateral-flow techniques. It is hoped that this approach will address the need for point-of-care (POC) testing in molecular diagnostics, eventually making it possible for clinicians to conduct rapid and inexpensive on-site testing for a variety of disease markers.

Roy R. Mondesire is project leader, Diane L. Kozwich is a former vice president for operations, Kristine A. Johansen is a senior scientist, John C. Gerdes is vice president for research and development, and Shannon E. Beard is director of business development at Xtrana Inc. (Denver). *This article is based on work supported by the Cooperative State Research, Education, and Extension Service, U.S. Department of Agriculture, under agreement nos. 99-33610-8149 and 98-33610-6345. Opinions, findings, conclusions, and recommendations expressed herein are those of the authors and do not necessarily reflect the view of the U.S. Department of Agriculture. The SCIP project was funded by the Advanced Technology Program and the engineering, by Ansys Diagnostics , Inc.*

Extraction and Capture

In the method developed by Xtrana Inc. (Denver), the immobilization of nucleic acid target is achieved through the use of the company's solid-phase nucleic acid binding material trademarked as XtraBind. This material has a number of unique properties that provide for rapid nucleic acid capture and purification. It can irreversibly bind both single-stranded DNA and RNA. Under optimal buffer conditions, capture of the nucleic acid target occurs immediately even when starting with low copy numbers. DNA thus bound to XtraBind is quite stable and can be stored for later analysis for at least 18 months. Nucleic acid captured using this material can be amplified directly on the solid phase using a variety of amplification strategies, including those requiring single-strand initiation. The material is commercially available in a kit format for use with blood (the XtraAmp whole blood DNA-extraction kit), and is capable of capturing single copies of genes from as little as 10 µl of blood. Studies are under way to examine the use of this material for nucleic acid extraction from several other types of clinical samples.

	Xtrana Method		
FSIS Method	Positive	Negative	Total
Positive	5	0	5
Negative	0	17	17
Total	5	17	22

Table I. Results of a study comparing the binding properties of the Food Safety and Inspection Service (FSIS) method to Xtrana's solid-phase nucleic acid binding material, XtraBind.

One study investigated the binding properties of the XtraBind solid-phase surface as compared with the Food Safety and Inspection Service (FSIS) method. As its target molecule,

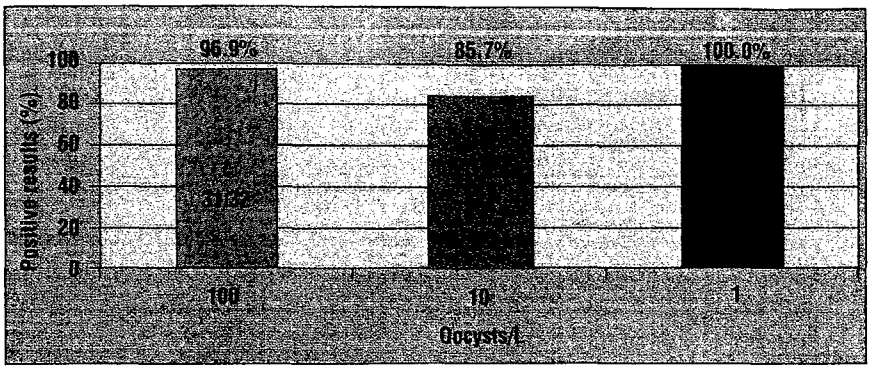


Figure 1. Detection of *Cryptosporidium parvum* oocysts in raw water samples. The bars represent positive lateral-flow results following the spiking of oocysts, extraction of RNA, and amplification by NASBA.

the study used RNA from *Escherichia coli* 0157:H7 amplified by means of nucleic acid sequence-based amplification (NASBA). Results of the study showed complete agreement with the FSIS method, and indicated that there was no cross-reactivity with related gram-negative microorganisms (see Table I). Another study of the solid-phase technology demonstrated that, after extraction and amplification, it could detect RNA from as little as one *Cryptosporidium parvum* oocyst per liter of water (see Figure 1). Other pathogenic microorganisms successfully adapted to this method include *Chlamydia trachomatis*, *Neisseria gonorrhea*, *Listeria monocytogenes*, and *Yersinia pestis*.

To selectively capture nucleic acid targets where very few copies are present in complex specimens, users can perform hybridization target selection with the specific oligonucleotide-derivatized XtraBind where the target nucleic acid is to be captured. In this approach, total nucleic acid is released from the test specimen, then diluted with a hybridization buffer. Hybridization capture makes it possible to select for low concentrations of specific target, even in the presence of high levels of other molecules. Proteins and other potential inhibitors of the nucleic acid amplification reaction are removed by washing.

Nested PCR employs two sets of amplification primers to conduct a dual amplification process that results in very high sensitivity.

Amplification

The technology developed by Xtrana can be adapted to a variety of target amplification techniques, including the widely used polymerase chain reaction (PCR) and related methods. PCR is based on the ability of DNA polymerase to copy a strand of DNA by elongation of complementary strands initiated from a pair of oligonucleotide primers.

Reverse transcriptase-PCR is used to amplify RNA targets. In this process, the reverse transcriptase enzyme is used to convert RNA to complementary DNA (cDNA), which can then be amplified using PCR. This method has proven useful for the detection of RNA viruses.

Nested PCR employs two sets of amplification primers to conduct a dual amplification process that results in very high sensitivity. In multiplexed PCR, two or more pairs of primers for different targets are introduced into the same reaction mixture. Here, there is simultaneous amplification of more than one unique DNA sequence present in a sample.

Isothermal nucleic acid amplification systems include the transcription-based amplification system (TAS) and its derivatives. Methods based on TAS include self-sustaining sequence replication (3SR), NASBA, and transcription-mediated amplification (TMA). Another non-PCR isothermal system is strand-displacement amplification (SDA). The ligase chain reaction (LCR), a probe amplification technique, has also been successfully used in diagnostics.

Detection Chemistries

A variety of chemistries are available for the detection and measurement of amplified target molecules. When colorimetric methods are used, results can be assessed visually or with the aid of a spectrophotometer. Labeling of the target is commonly accomplished by using enzymes such as horseradish peroxidase. Two of the common chromogenic substrates for peroxidase are 3,3′,5,5′-tetramethylbenzidine (TMB) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS).

Detection techniques based on the use of fluorescent labels are also useful in clinical diagnostics.[6] Fluorometric assays are more sensitive than colorimetric ones and can be used to detect or measure lower levels of nucleic acid. However, fluorometric assays have the disadvantages that they involve more-complex procedures and must be assessed using instrumentation. The use of traditional fluorescent compounds is further complicated by the background interference that can be caused by light scattering and the intrinsic fluorescence of sample components. To overcome such limitations related to background interference, assay developers can design tests to make use of time-resolved fluorescence. The technique of time-resolved fluorescence is based on the principle that some lanthanides, such as europium (Eu^{3+}), form fluorescent chelates with certain organic ligands. These fluorophores have very large Stokes' shifts and decay times (200 nm and >500 ns, respectively). Time-resolved fluorescence takes advantage of these long decay times and

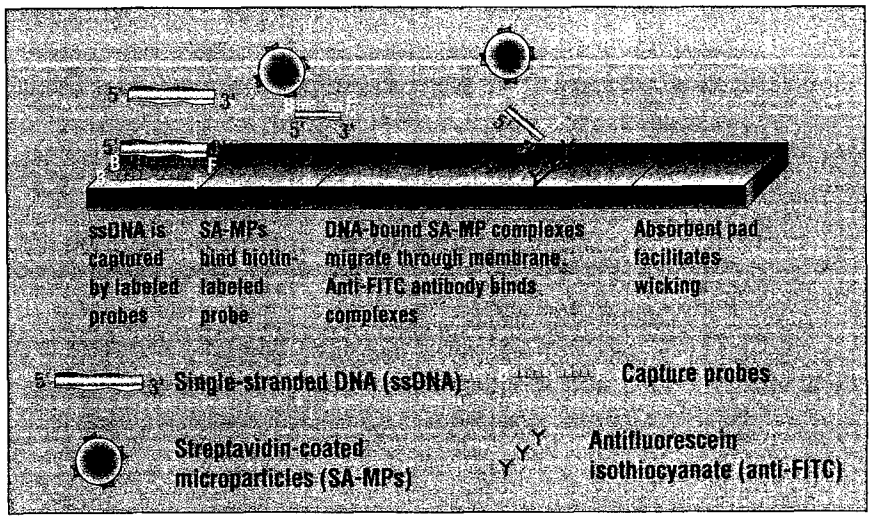


Figure 2. Schematic of Xtrana's lateral-flow method for detection of amplified single-stranded DNA intermediates following amplification. The migration of the microsphere labeled nucleic acid complexes are arrested at the antifluorescein isothiocyanate stripe, where the reaction is observed by the user.

large Stokes' shifts, making them qualifiers for the fluorescent signal to be recorded. Thus, any short-lived fluorescent background signal or scattered excitation radiation can be readily filtered for elimination, and the desired fluorescent signals can be measured under conditions that are virtually without background interference.

In chemiluminescent assays, luminescent compounds emit light during the course of a chemical reaction. The labels used for such assays are commonly luminol derivatives or acridinium esters. The kinetics of assays using chemiluminescence are very fast, and light is emitted within seconds of substrate oxidation. In an electrochemiluminescence (ECL) technique, a ruthenium metal chelate and tripropylamine are utilized.[7,8] Both of these molecules become oxidized at the surface of an electrode, where they react to form an excited state of ruthenium that decays, releasing a photon at 620 nm.

Detection techniques using biosensors are also gaining acceptance among the makers of nucleic acid diagnostics. Biosensors are analytical devices in which a molecule of biological origin serves as the chemical recognition element (e.g., an antibody or enzyme), and is measured when it comes into contact with a physical transducer or detector.[9] A wide variety of such biosensors have been developed, including biocatalytic, electrochemical, optical, mass-detection, amperometric, pharmacological, and immunochemical sensors.[10,11] In the field of clinical diagnostics, the application of immunological biosensors has gathered considerable impetus, with a rapid influx of new applications now under development. Some DNA biosensors utilize single-stranded DNA immobilized on quartz optical fibers, piezoelectric crystals, or amperometric electrodes. Unfortunately, such biosensor detection technologies are relatively complex, expensive, and require instrumentation in the vast majority of cases.

Lateral-Flow Detection

Although the detection and measurement technologies described above are suitable for use in nucleic acid assays, most require instrumentation that increases the overall costs of such testing. To eliminate this drawback, Xtrana has focused its efforts on developing a detection platform based on lateral-flow principles utilizing dyed microsphere labels. The method is sensitive, inexpensive, and easy to perform.

To perform the assay, the operator adds biotinylated and fluoresceinated oligonucleotide detection probes to the amplified reaction mixture. At this time, if the target is present, the two sets of probes will specifically hybridize with the same strand of the target DNA or RNA molecule. When mixed with streptavidin-coated dyed microspheres, the biotinylated portion of the probe will bind to the streptavidin. The resultant complexes are then applied to a nitrocellulose membrane containing an immobilized antifluorescein isothiocyanate antibody line.

Migration of the complexes occurs through the membrane until the antibody binds the fluoresceinated portion of the probe. This interaction arrests further migration of the microsphere-haptenized duplexes and rapid accumulation of these complexes occurs at the antibody line (see Figure 2). The unaided eye can easily detect this result. Figure 3 shows the correlation between agarose gel electrophoresis and the lateral-flow technique. The lateral-flow test is several logs more sensitive than gel electrophoresis, and the results are revealed within two minutes after the addition of reaction products to the membranes.

Lateral-flow detection methods are economical and do not require instrumentation. This translates into a drastic reduction in cost for nucleic acid–based testing. These methods are usually single-step assays that do not require multiple washing or long incubation steps.

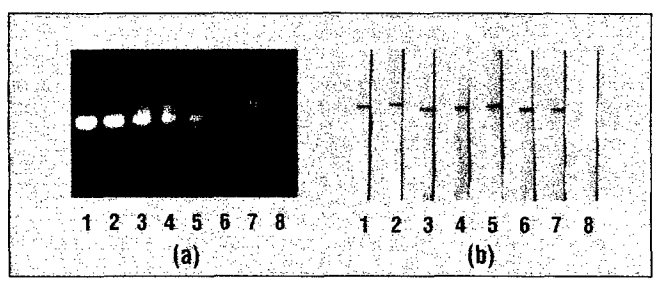


Figure 3. Agarose gel electrophoresis (a) and detection by lateral flow (b) of specific RNA product after NASBA amplification of target captured using XtraBind. Lanes 1 through 7 represent 10-fold dilutions of product starting at a high copy of specifically amplified RNA. Lane 8 is a no-target control.

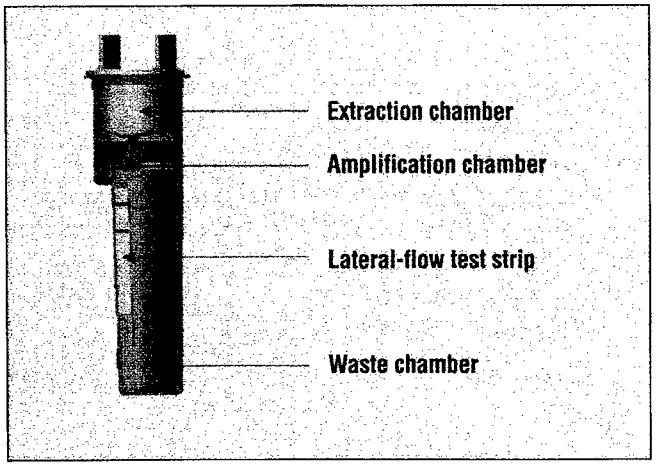


Figure 4. Prototype of the self-contained integrated particle (SCIP) device illustrating its essential components. Extraction, amplification, and detection are achieved in a self-contained fashion through a series of simple fluid-transfer processes.

Containment

As a containment vessel for its lateral-flow molecular assays, Xtrana has developed a closed, self-contained device called the self-contained integrated particle (SCIP), which combines all the steps required to perform DNA or RNA testing (see Figure 4). The sample is introduced into one end of the device and a series of simple manipulations moves the sample to other compartments of the device where extraction, amplification, and detection occur automatically. This closed system prevents contamination and eliminates the need for expensive equipment.

The SCIP device integrates XtraBind for nucleic acid extraction with immunodiagnostic detection technology. Initial experiments using SCIP prototypes have produced detection sensitivities that meet currently available DNA testing requirements. This SCIP device, incorporating microsphere lateral-flow detection, removes many of the roadblocks of cost and complexity associated with the technological transfer and adaptation of nucleic acid–based testing to the end-user. Furthermore, it should produce a significant shift of certain types of diagnostic testing from centralized urban reference laboratories to rural settings or POC testing.

Conclusion

The current market for in vitro diagnostics is approximately $20 billion worldwide. The U.S. portion is estimated at approximately $8 billion, with clinical chemistry and immunoassay testing making up approximately 45%.[12] However, molecular technologies are rapidly forging their way to the forefront of the industry, and with a current market share of 3%, DNA- and RNA-probe diagnostics constitute the fastest growing segment of the clinical diagnostics market.

Some analysts have suggested that today's competitive market will demand that new molecular technologies fulfill the need for a broad panel of assays, automated systems, and high throughput.[13] However, the complexity and high cost of molecular systems designed to meet all such needs may prohibit their use in some laboratories. A formidable challenge for manufacturers in the rapidly expanding nucleic acid diagnostic market will be to produce easy-to-use, inexpensive devices with high-performance characteristics. The R&D efforts at Xtrana are directed toward achieving these requirements.

The development of molecular diagnostic technologies in this decade will be influenced by innovations in chemistry, molecular biology, immunology, automation, and data management. At the same time, small and inexpensive POC devices will continue to play a major role in providing accurate and objective results in doctors' offices as well as alternative-care settings. Manufacturers of molecular diagnostics are in an exciting early phase of this technology and have new challenges that offer the opportunity to assist in providing the highest quality medical care worldwide.

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Intergen Company

PerSeptive Biosystems

Promega Corporation

Sigma Diagnostics

AACC

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46

Nucleic acid extraction, amplification, and visual detection in a contained, closed system.

Mondesire R, Beard S, Gerdes J, Kozwich D, Woronoff S. Xtrana, Inc., Denver, CO.

Molecular Innovations, Inc. has developed a new approach to nucleic acid testing for forensics and paternity identity testing, infectious human disease testing (including bacterial warfare agents), the detection of food and environmental contamination, research and other clinical applications. Broader future applications include test systems for genetic predisposition to disease and the human diagnostic markets. A system has been created for integrating nucleic acid extraction, amplification, and detection into a single, self-contained field-usable device. This system, referred to as SCIP (for "Self-Contained Integrated Particle" assay), is amenable to blood, water, tissue, food, urine, and other specimens.

Samples are lysed in the presence of a solid phase nucleic acid binding matrix, Xtra Bind™, which irreversibly captures the nucleic acid and allows for direct amplification on the solid phase. The lysate and wash reagents are collected in a waste chamber, and the bound target is then amplified by PCR or other amplification schemes. There are also a number of isothermal amplification strategies which are used in this system and do not require a thermocycler. The sequence-specific amplification product is detected by a lateral flow assay. Amplification reaction mixture wicks onto a nitrocellulose membrane that contains two distinctly labeled oligos that serve as probes to the target nucleic acid. The bi-functionally haptenized product migrates further along the membrane by capillary action and dyed microparticles are picked up by one of the haptens. The product is then captured by an antibody line on the membrane specific to the second hapten. The result is a visible blue line, generated within 1-2 minutes after amplification.

Current projects, funded by grants and contracts, using the SCIP technology include detection tests for: *Yersinia pestis, Cryptosporidium parvum, E. coli* 0157H, *Listeria monocytogenes*, coliforms, and *Chlamydia*. The detection limit for many of these organisms is at the single organism level. The detection limit of the lateral flow assay is currently 10^7 copies of target sequence, post-amplification.

Appendix VII Patents

The United States of America

The Commissioner of Patents and Trademarks

Has received an application for a patent for a new and useful invention. The title and description of the invention are enclosed. The requirements of law have been complied with, and it has been determined that a patent on the invention shall be granted under the law.

Therefore, this

United States Patent

Grants to the person(s) having title to this patent the right to exclude others from making, using, offering for sale, or selling the invention throughout the United States of America or importing the invention into the United States of America for the term set forth below, subject to the payment of maintenance fees as provided by law.

If this application was filed prior to June 8, 1995, the term of this patent is the longer of seventeen years from the date of grant of this patent or twenty years from the earliest effective U.S. filing date of the application, subject to any statutory extension.

If this application was filed on or after June 8, 1995, the term of this patent is twenty years from the U.S. filing date, subject to any statutory extension. If the application contains a specific reference to an earlier filed application or applications under 35 U.S.C. 120, 121 or 365(c), the term of the patent is twenty years from the date on which the earliest application was filed, subject to any statutory extension.

Acting Commissioner of Patents and Trademarks

Attest

NOTICE

If the application for this patent was filed on or after December 12, 1980, maintenance fees are due three years and six months, seven years and six months, and eleven years and six months after the date of this grant, or within a grace period of six months thereafter upon payment of a surcharge as provided by law. The amount, number and timing of the maintenance fees required may be changed by law or regulation. Unless payment of the applicable maintenance fee is received in the Patent and Trademark Office on or before the date the fee is due or within a grace period of six months thereafter, the patent will expire as of the end of such grace period.

US005955351A

United States Patent [19]

Gerdes et al.

[11] Patent Number: 5,955,351

[45] Date of Patent: Sep. 21, 1999

[54] **SELF-CONTAINED DEVICE INTEGRATING NUCLEIC ACID EXTRACTION AMPLIFICATION AND DETECTION**

[76] Inventors: **John C. Gerdes**, 375 Steele St., Denver, Colo. 80206; **Lynn D. Jankovsky**, 19 Belleview La., Greenwood Village, Colo. 80121; **Diane L. Kozwich**, 4915 S. Huron St., Englewood, Colo. 80110

[21] Appl. No.: **08/679,522**

[22] Filed: **Jul. 12, 1996**

Related U.S. Application Data

[60] Provisional application No. 60/000.885, Jul. 13, 1995.

[51] **Int. Cl.**[6] **C12M 1/00**; G01N 15/06; G05D 16/00; C12N 15/00

[52] **U.S. Cl.** **435/287.2**; 422/68.1; 422/112; 436/810; 935/76; 935/77; 935/78; 435/6; 435/91.2

[58] **Field of Search** 435/287.2, 6, 91.2; 422/68.1, 112; 436/810; 935/76, 77, 78

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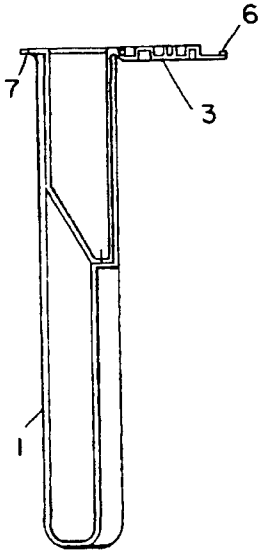
Primary Examiner—W. Gary Jones
Assistant Examiner—Ethan Whisenant
Attorney, Agent, or Firm—Julie L. Bernard

[57] **ABSTRACT**

A self-contained device is described that integrates nucleic acid extraction, specific target amplification and detection into a single device. This integration permits rapid and accurate nucleic acid sequence detection. The invention may be used, for example, in the screening for nucleic acid sequences which may be indicative of genetic defects or contagious diseases, as well as for monitoring efficacy in the treatment of contagious diseases.

9 Claims, 11 Drawing Sheets



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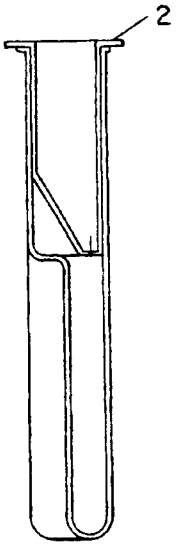

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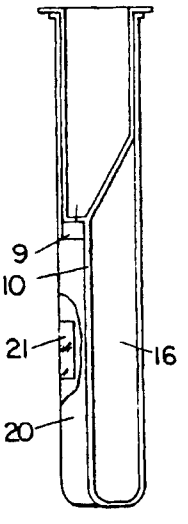

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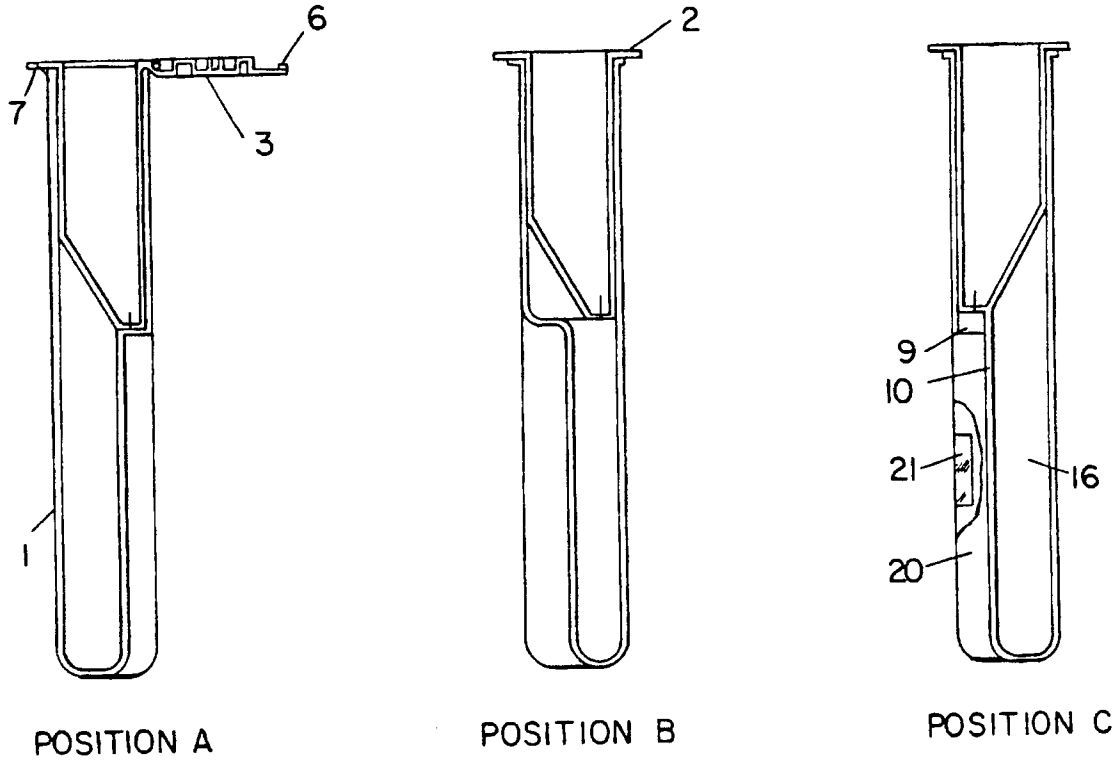
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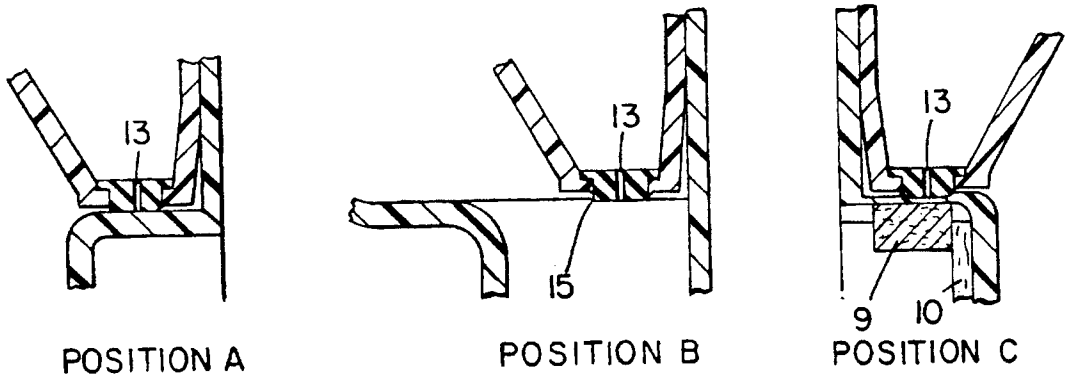
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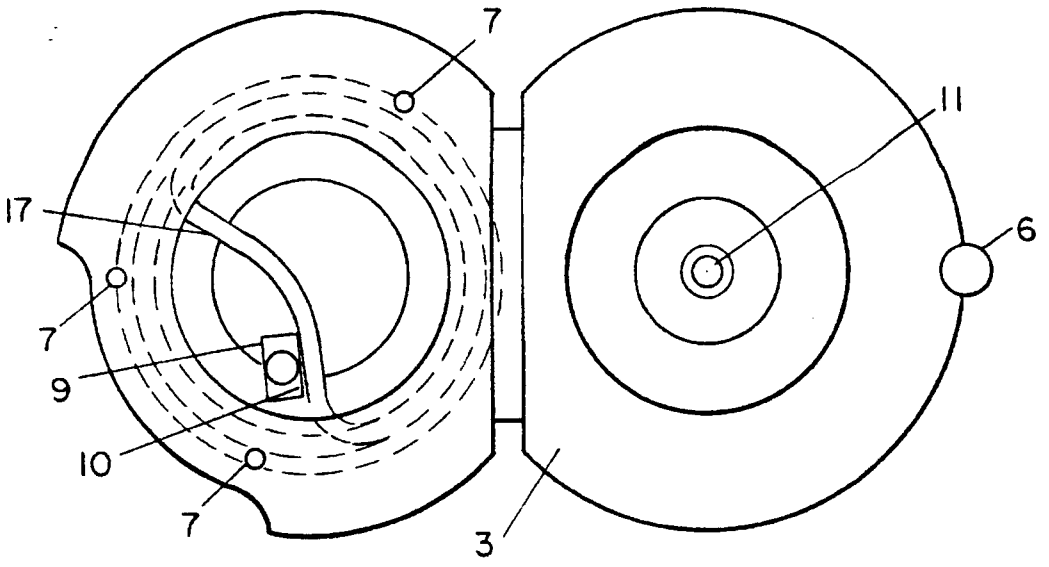


FIG. 3

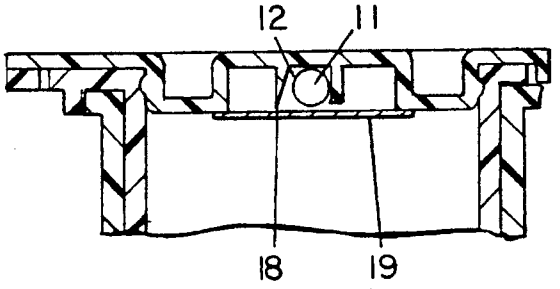
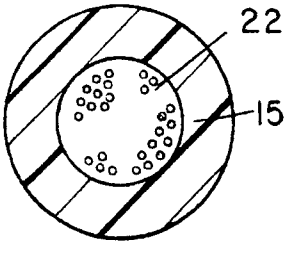


FIG. 4



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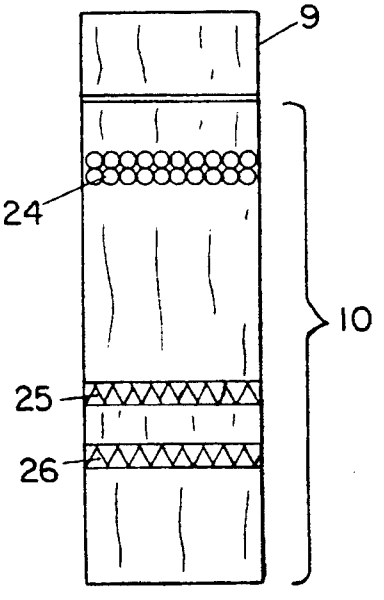

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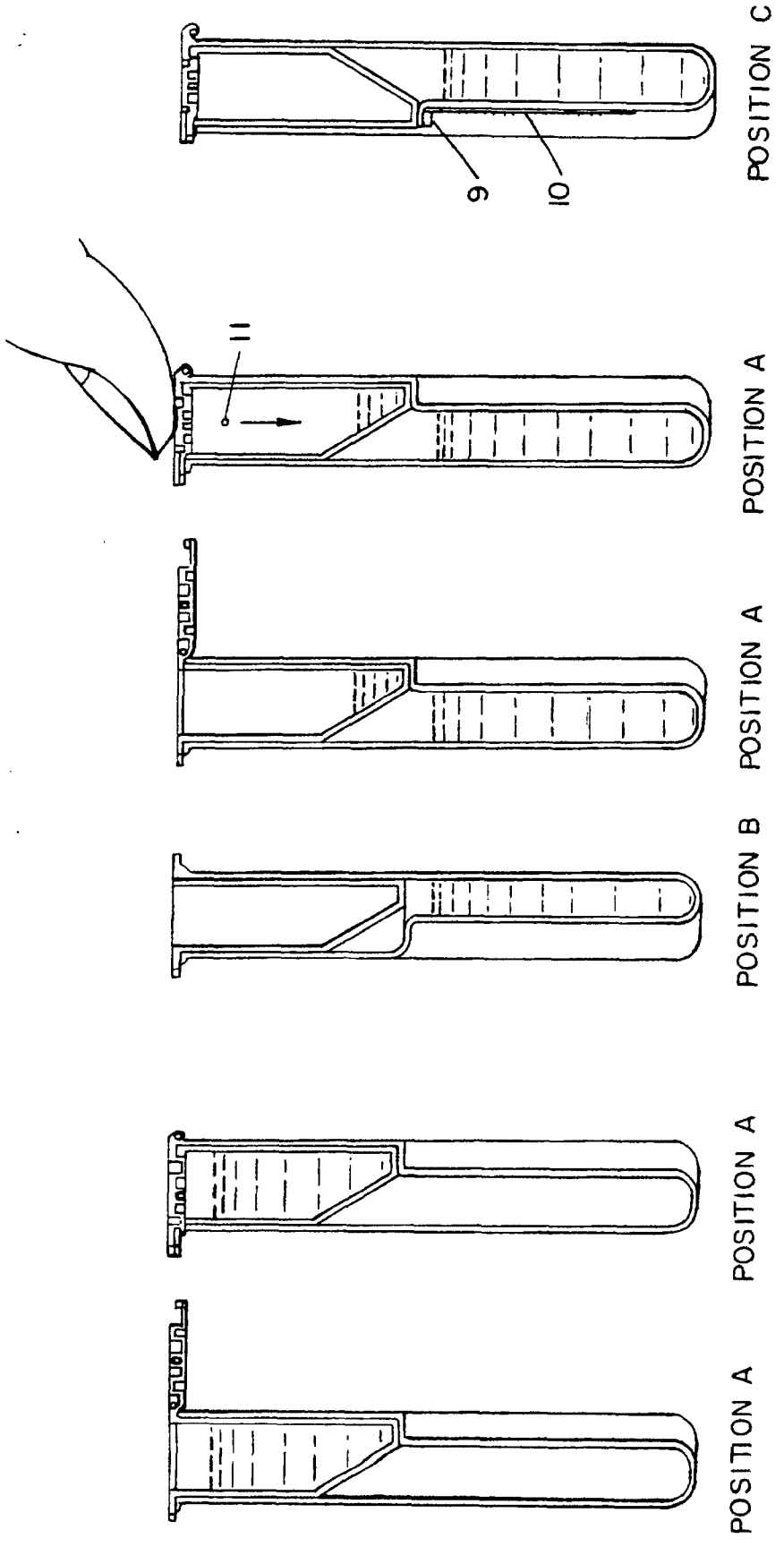
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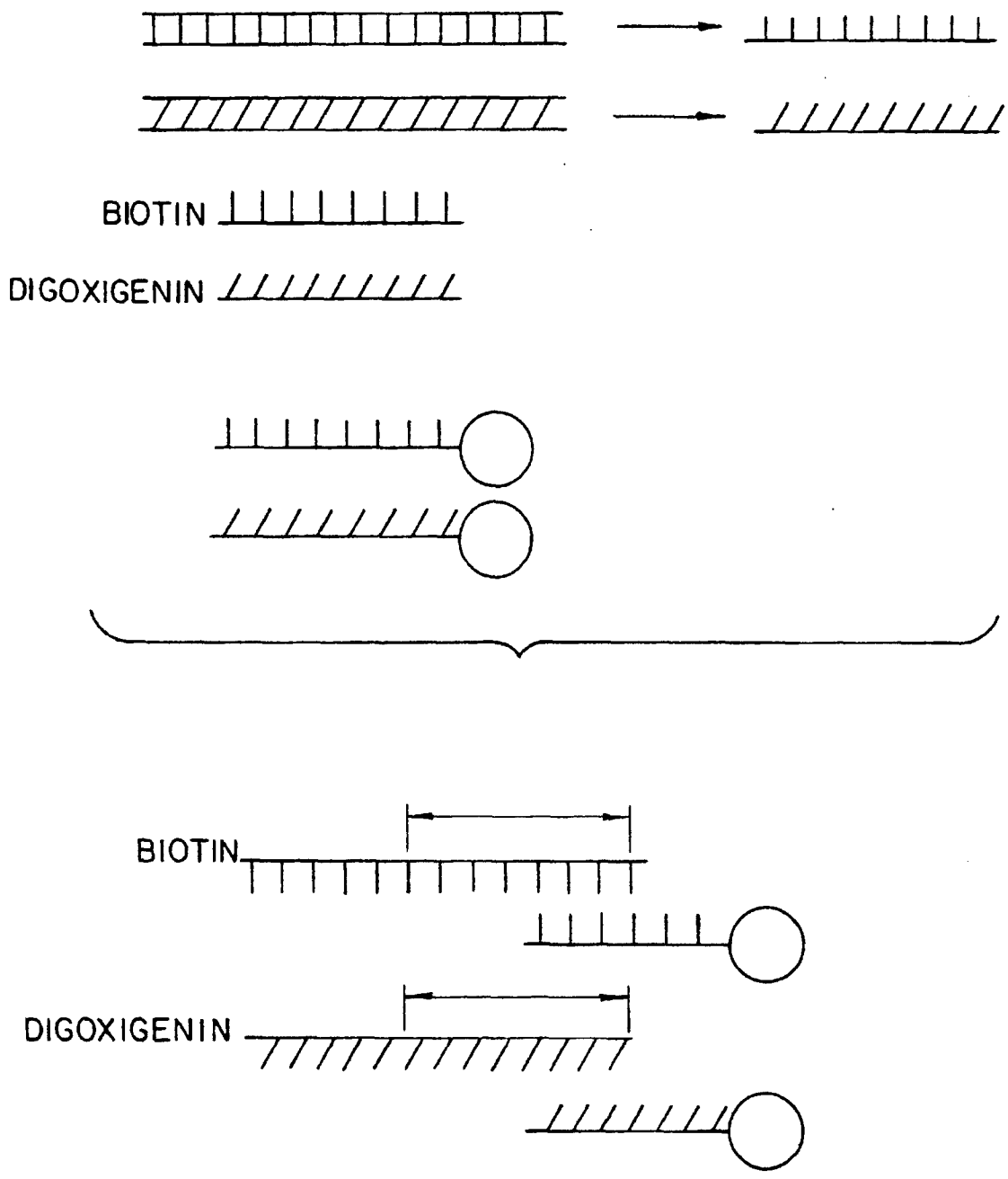
FIG. 7



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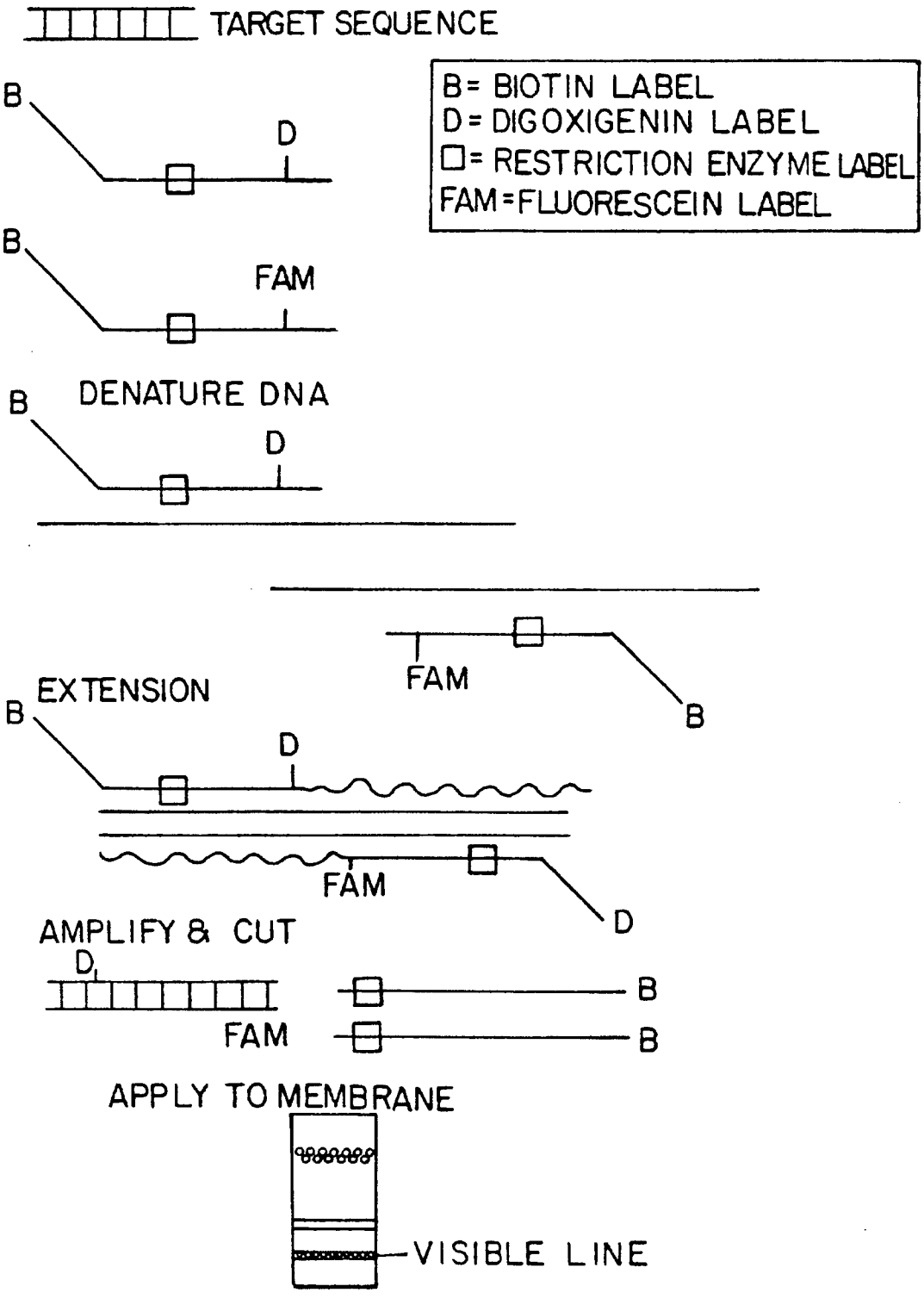

TARGET SEQUENCE
B= BIOTIN LABEL
D= DIGOXIGENIN LABEL
□= RESTRICTION ENZYME LABEL
FAM=FLUORESCEIN LABEL
B
D
B
FAM
B
DENATURE DNA
D
FAM
B
EXTENSION
B
D
FAM
D
AMPLIFY & CUT
D
B
FAM
B
APPLY TO MEMBRANE
VISIBLE LINE

FIG. 9

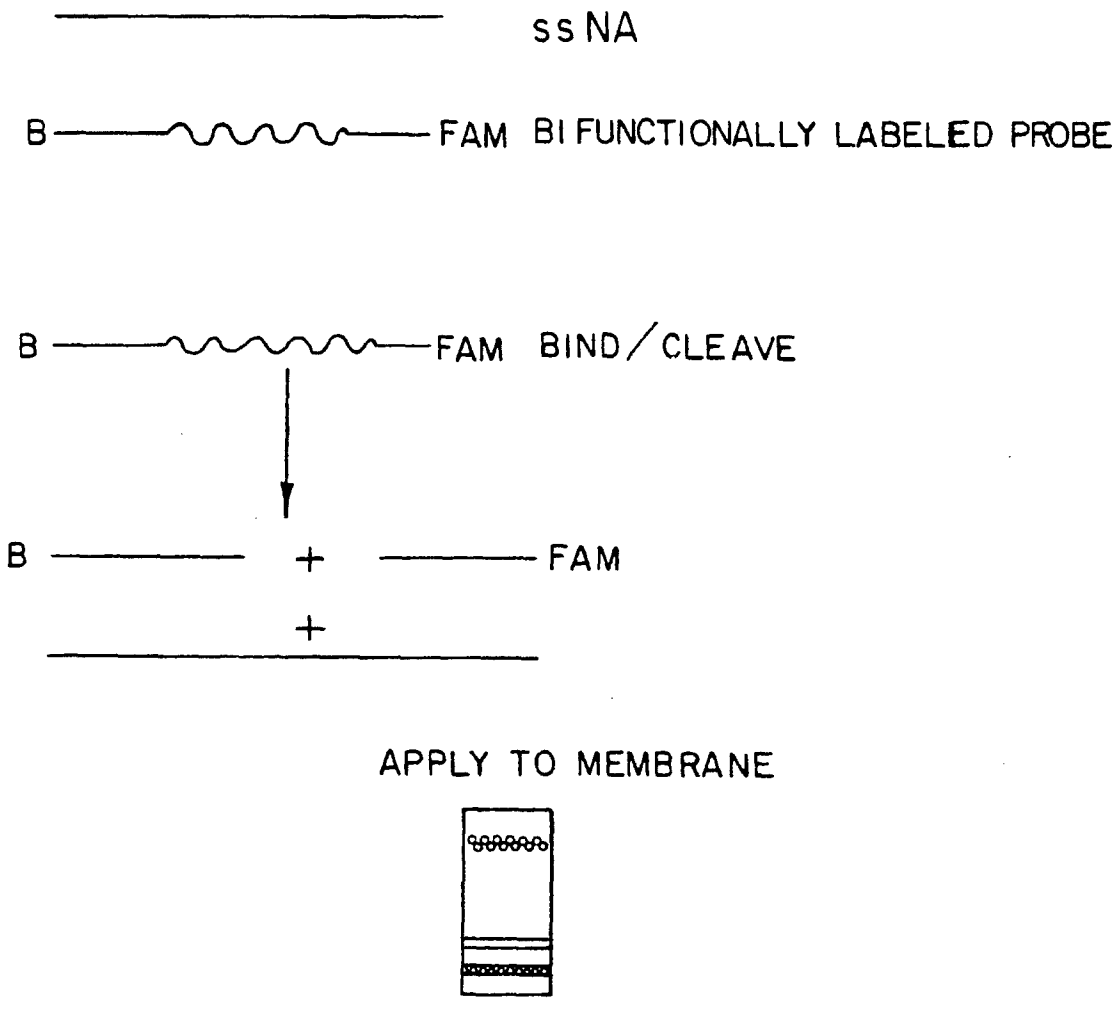

ssNA
B
FAM BIFUNCTIONALLY LABELED PROBE
B
FAM BIND/CLEAVE
B
FAM
APPLY TO MEMBRANE

FIG. 10

SDA FROM GEL

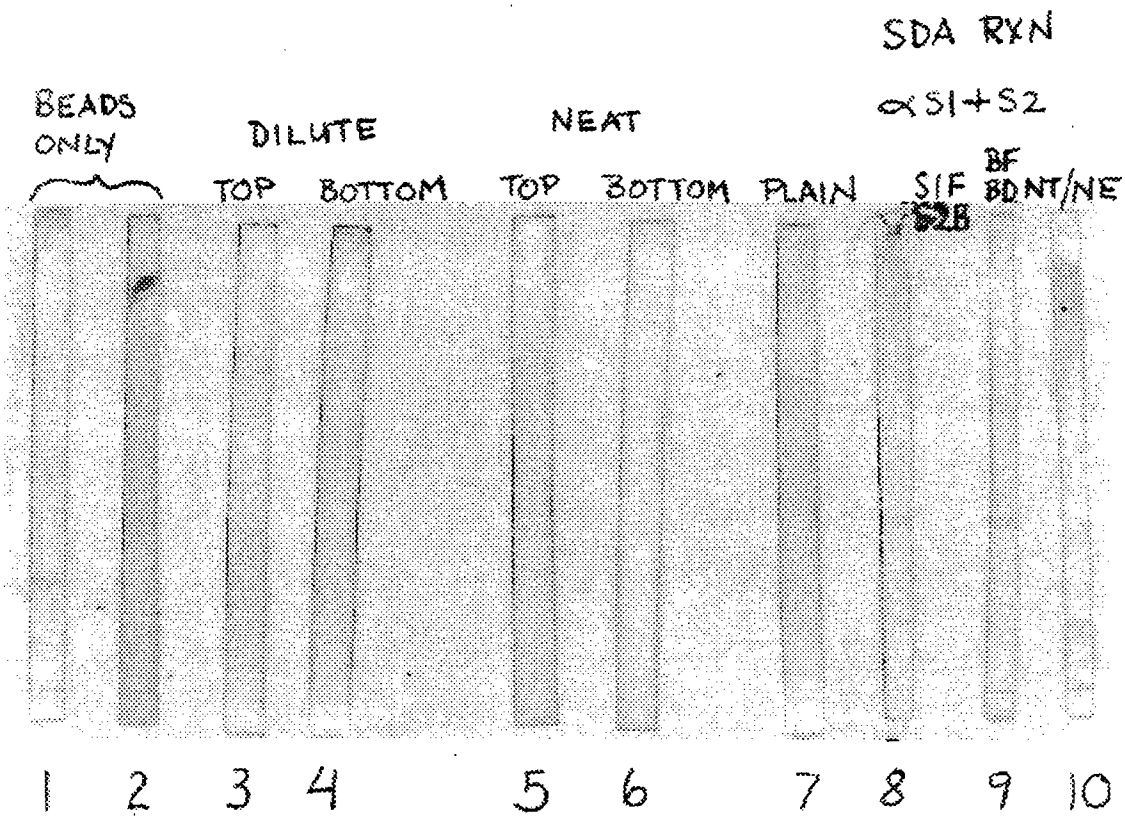


FIG. 11

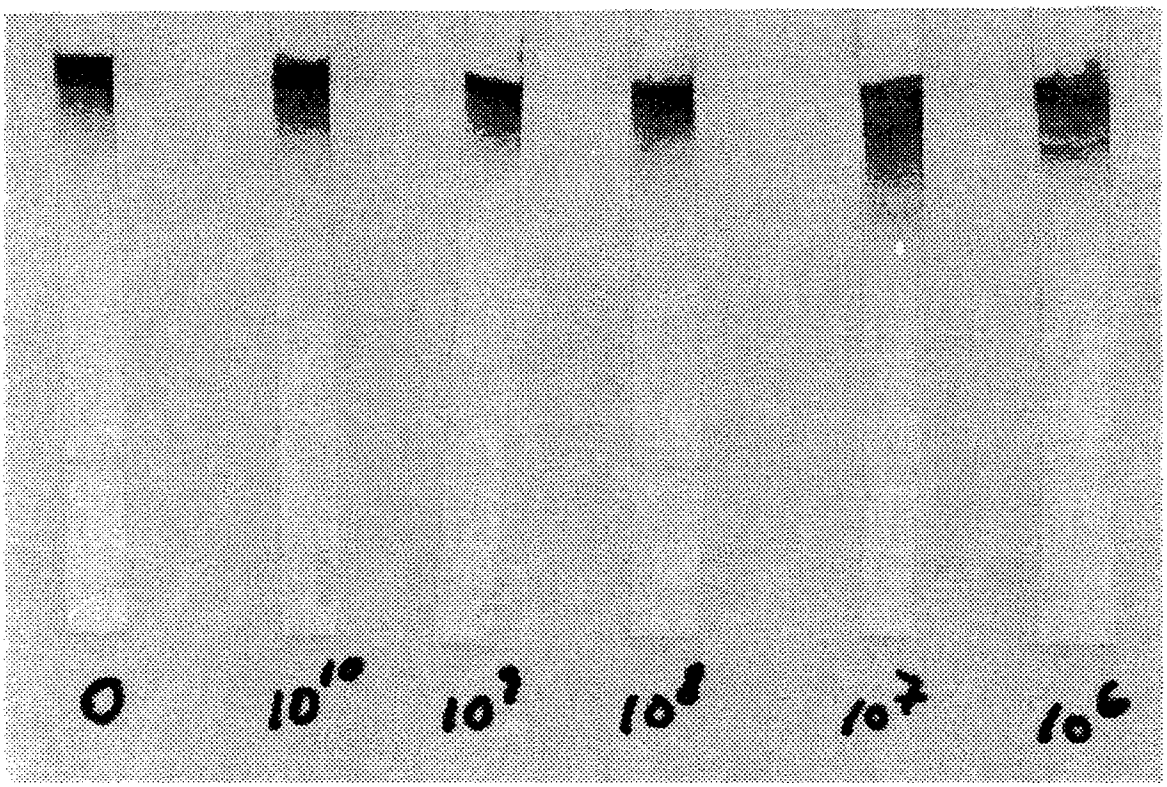


FIG. 12

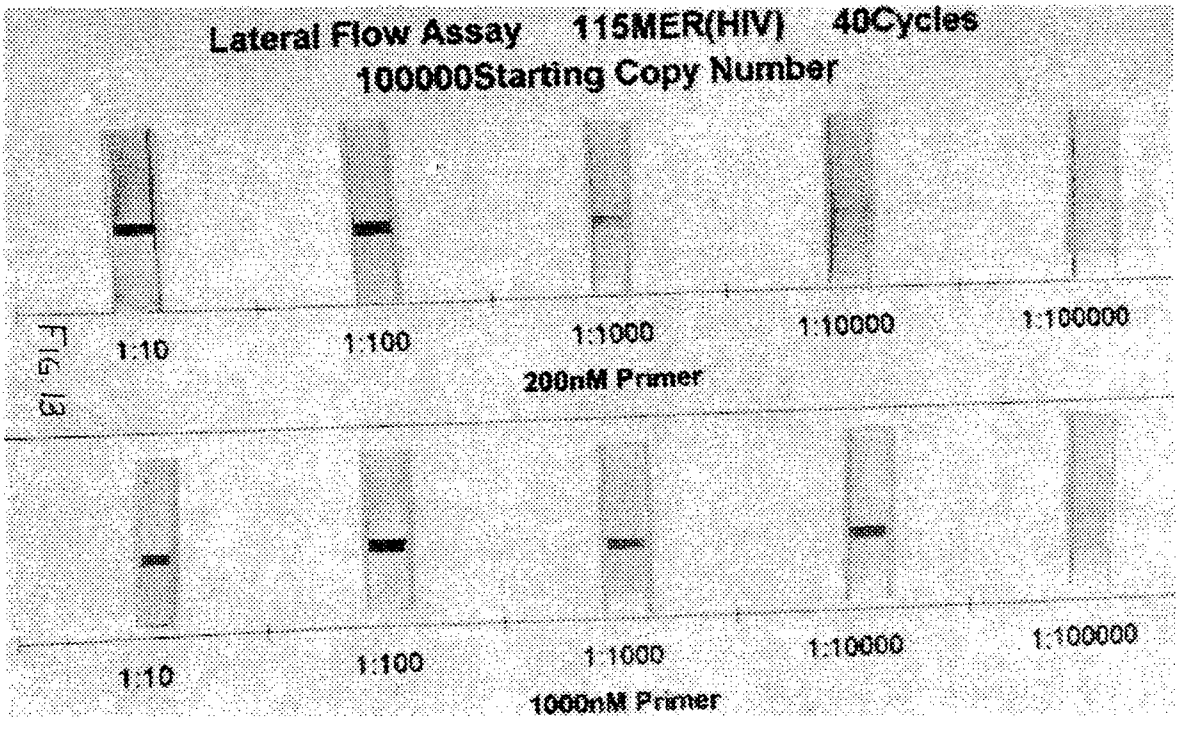
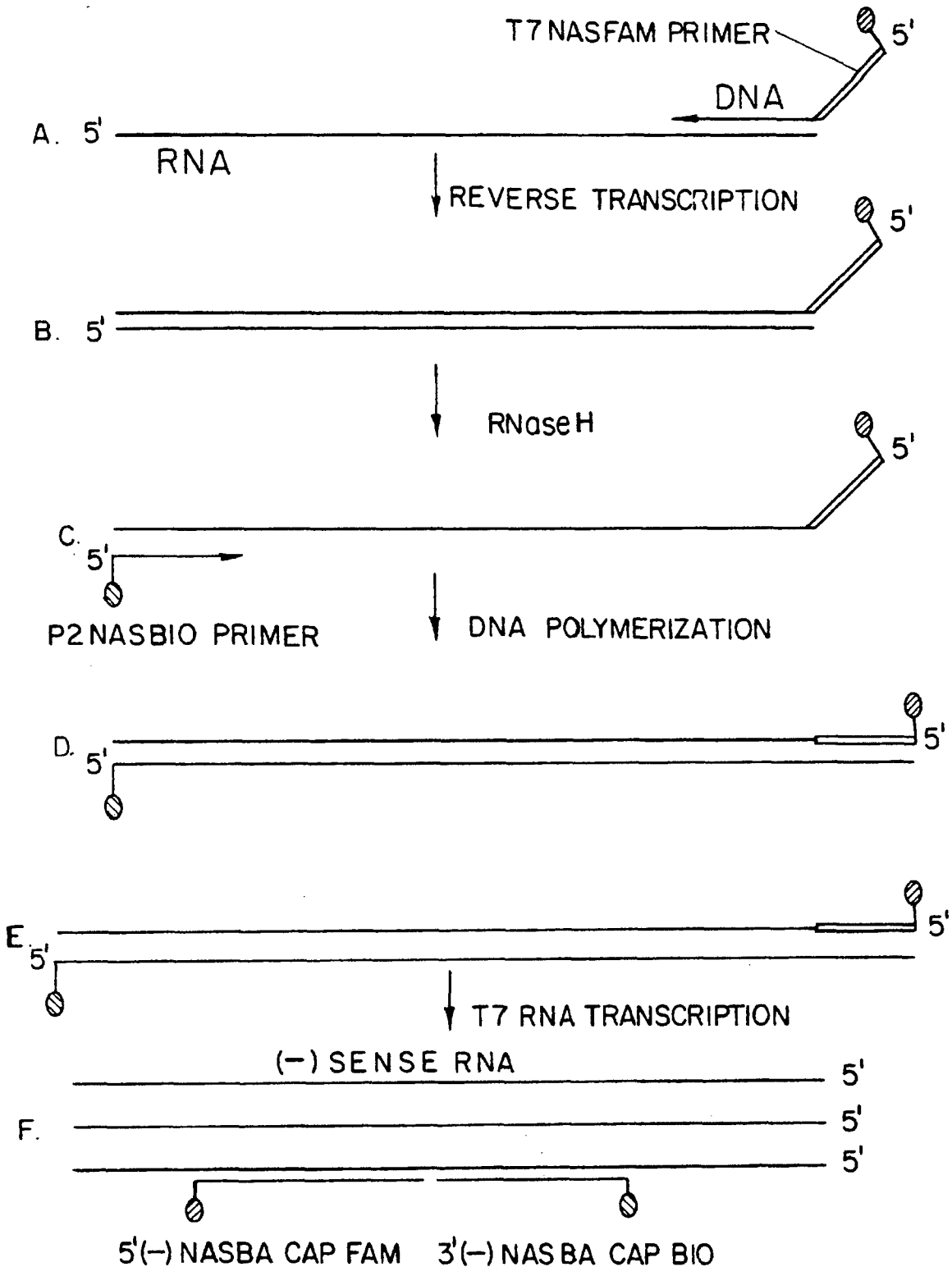

Lateral Flow Assay 115MER(HIV) 40Cycles
100000Starting Copy Number
1:10
1:100
1:1000
1:10000
1:100000
200nM Primer
1:10
1:100
1:1000
1:10000
1:100000
1000nM Primer

FIG. 13


T7 NASFAM PRIMER
5'
DNA
A. 5'
RNA
REVERSE TRANSCRIPTION
5'
B. 5'
RNaseH
5'
C.
5'
P2 NASBIO PRIMER
DNA POLYMERIZATION
D.
5'
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E
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T7 RNA TRANSCRIPTION
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FIG. 14

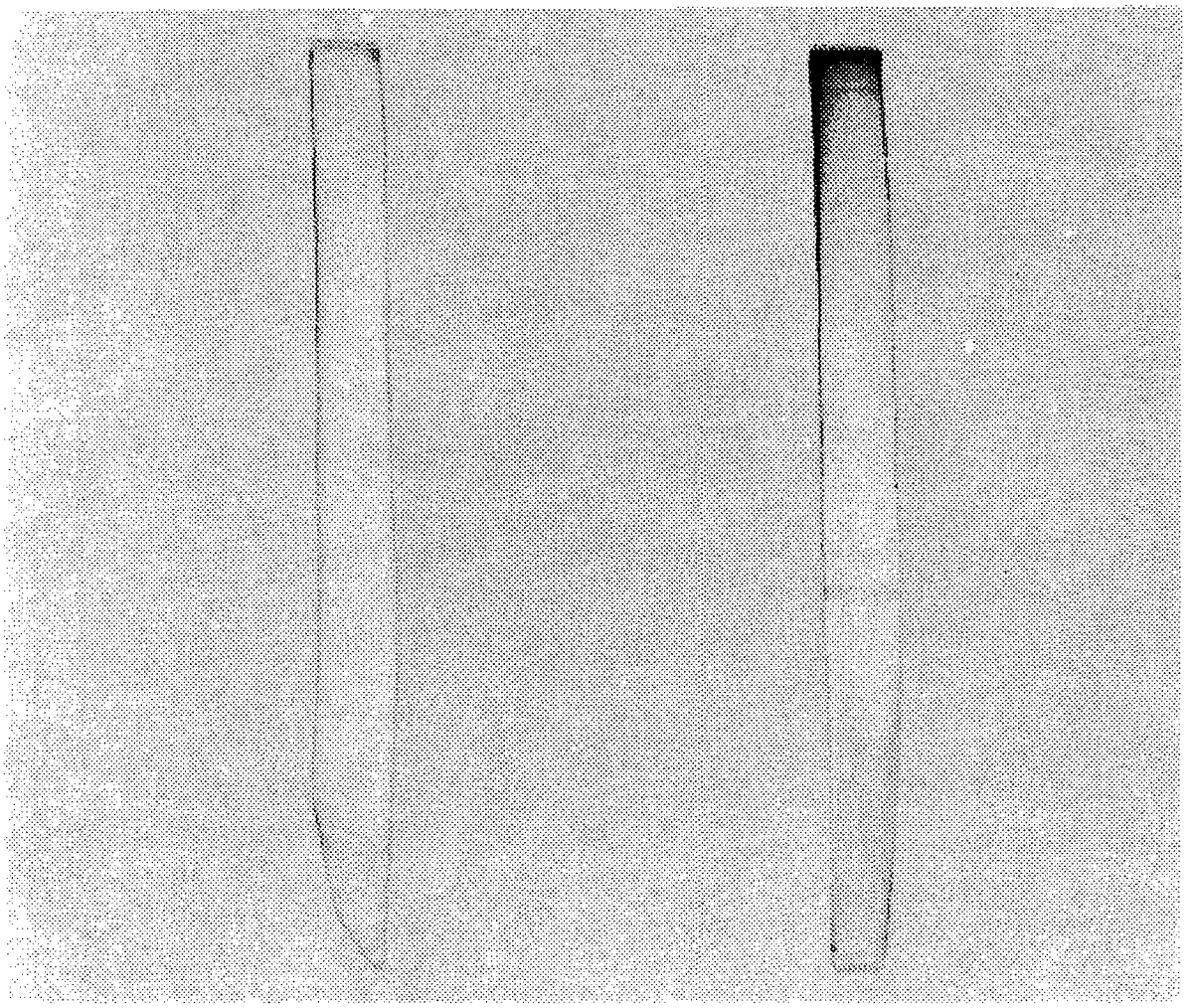

FIG. 15

SELF-CONTAINED DEVICE INTEGRATING NUCLEIC ACID EXTRACTION AMPLIFICATION AND DETECTION

RELATED APPLICATIONS

This application claims priority to provisional patent application Ser. No. 06/000,885, filed Jul. 13, 1995.

FIELD OF INVENTION

This invention relates to the general fields of molecular biology and medical science, and specifically to a method of extracting nucleic acid, amplifying specific target sequences, and detecting amplified nucleic acid sequences in a self-contained device. This application, thus, describes a self-contained device capable of rapid and accurate detection of target nucleic acid sequences.

BACKGROUND AND PRIOR ART

The use of nucleic acid probe tests based on hybridization in routine clinical laboratory procedures is hindered by lack of sensitivity. The ability to amplify nucleic acids from clinical samples has greatly advanced nucleic acid probe technology, providing the sensitivity lacking in earlier versions of non-isotopic assays. Sensitivity afforded by oligonucleotide probe tests utilizing nucleic acid amplification now exceeds that of any other method. Nucleic acid amplification procedures can detect a single copy of a specific nucleic acid sequence. Routine detection and identification of specific gene sequences have extremely broad application in a number of settings and industries.

The major barrier for the transfer of technology to routine field testing is the absence of an economical and easy-to-use system or apparatus. In order to compete in today's cost conscious environment, genetic based testing must provide for high throughput, while incorporating adequate controls and safeguards to prevent false positive results due to sample cross-contamination.

Current technology involves several steps, although recent developments are directed toward automating systems for detection of the amplified target sequence. The first step, extraction of nucleic acids, is accomplished in a variety of ways, for example, phenol extraction, chaotropic reagent extraction, chromatographic purification (Qiagen, WO 95/01359, purification on silica membranes, specifically incorporated herein) and ultracentrifugation (Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), specifically incorporated herein). Phenol is a well-established health hazard and requires special handling for waste removal. The extraction method is also tedious and labor intensive. Ultracentrifugation often requires the use of expensive and hazardous chemicals as well as the use of sophisticated and costly equipment. The process often requires long run times, sometimes involving one or more days of centrifugation. The easiest and fastest method is separation using chromatography purification.

The second step, the amplification of the target nucleic acid, employs a variety of enzymes known as polymerases and ligases. Polymerase chain reaction (PCR) is the most commonly used amplification technique. The general principles and conditions for amplification of nucleic acids using PCR are quite well known in the art; the details of which are provided in numerous references including U.S. Pat. No. 4,683,195, U.S. Pat. No. 4,683,202 and U.S. Pat. No. 4,965,188, all to Mullis et al., all of which are specifically

incorporated herein. Thus, the details of PCR technology are not included herein. Other approaches include ligase chain reaction, Qβ replicase, strand displacement assay, transcription mediated iso CR cycling probe technology and nucleic acid sequence-based amplification (NASBA).

A current protein detection technology for antigen-antibody assays involves the use of microparticles. Furthermore, a variety of microparticle strategies for dipstick detection antigen-antibody assays are currently available, for example, a currently marketed at-home pregnancy test (U.S. Pat. No. 5,141,850 to Cole et al., specifically incorporated herein). Such tests use dyed particles that form a visible line following a specific antigen-antibody reaction. The instant invention is accomplished by hybridization of amplicons to capture oligonucleotides bound to microparticles. That is, the invention disclosed herein detects nucleic acid amplicons.

The detection of amplified nucleic acid for clinical use relies largely on hybridization of the amplified product and detection with a probe labeled with a variety of enzymes and luminescent reagents. U.S. Pat. No. 5,374,524 to Miller, specifically incorporated herein, describes a nucleic acid probe assay that combines nucleic acid amplification and solution hybridization using capture and reporter probes. These techniques require multiple reagents, several washing steps, and specialized equipment for detection of the target nucleic acid. Moreover, these techniques are labor intensive and require technicians with expertise in molecular biology.

The use of probes comprised of oligonucleotide sequences bound to microparticles is well known and illustrated in prior art. The mechanism for attachment of oligonucleotides to microparticles in hybridization assays and for the purification of nucleic acids is also well known. European Patent No. 200133, specifically incorporated herein, describes the attachment of oligonucleotides to water-insoluble particles less than 50 micrometers in diameter used in hybridization assays for the capture of target nucleotides. U.S. Pat. No. 5,387,512 to Wu, specifically incorporated herein, describes the use of oligonucleotide sequences covalently bound to microparticles as probes for capturing PCR amplified nucleic acids. U.S. Pat. No. 5,328,825 to Findlay, specifically incorporated herein, also describes an oligonucleotide linked by way of a protein or carbohydrate to a water-insoluble particle. The oligonucleotide probe is covalently coupled to the microparticle or other solid support. The sensitivity and specificity of all of the above-reference patents is based on hybridization of the oligonucleotide probe to the target nucleic acid.

The use of incorporated non-radioactive labels into the amplification reactions for the detection of nucleic acids is also well known in the art. Nucleic acids modified with biotin (U.S. Pat. No. 4,687,732 to Ward et al.; European Patent No. 063879; both specifically incorporated herein), digoxin (European Patent No. 173251, specifically incorporated herein) and other haptens have also been used. For example, U.S. Pat. No. 5,344,757 to Graf, specifically incorporated herein, uses a nucleic acid probe containing at least one hapten as label for hybridization with a complementary target nucleic acid bound to a solid membrane. The sensitivity and specificity of these assays is based on the incorporation of a single label in the amplification reaction which can be detected using an antibody specific to the label. The usual case involves an antibody conjugated to an enzyme. Furthermore, the addition of substrate generates a calorimetric or fluorescent change which can be detected with an instrument.

Still, the above-described approaches are labor intensive with many steps and washes; require special and costly

equipment for the detection of the target nucleic acid; require trained staff; and take several hours to complete. Several patents have issued which deal with automation of the processes of amplification and subsequent detection of the amplicon. These patents use specialized equipment and are still based on the principle of hybridization and immunoassay technology. For example, European Patent No. 320308, specifically incorporated herein, describes a system detecting target nucleic acids amplified by the ligase chain reaction.

Automated approaches eliminate the need for specially trained personnel, however, the cost of the equipment is very high and the possibility of contamination still exists since many samples will be processed by the same equipment. To eliminate the issue of contamination, it is necessary to integrate the three steps outlined above. The self-contained device disclosed herein accomplishes this goal by integrating existing nucleic acid extraction and isothermal amplification technologies with an innovative detection strategy.

The invention described herein provides for the rapid and accurate detection of amplified nucleic acid sequences using a self-contained device. The possibility of contamination is eliminated because of the "throw away" approach described herein. Elimination of cross contamination opens the door to mass screening including automation. The high sensitivity of the analysis allows for the early detection of disease and an opportunity for early treatment. The present invention diagnoses the presence of infectious diseases of genetic, bacterial or viral origin. Analysis by this invention can monitor the efficacy of treatment, for example, to monitor HIV virus in the plasma of patients undergoing therapy. Analysis, according to the invention disclosed herein, is easy, requiring little expertise in the art of molecular biology. The cost is significantly less than other methods currently in use to detect amplified nucleic acids. The time frame for detecting an amplified sequence is reduced drastically. There is no danger from potentially hazardous chemicals. The analysis does not require special waste disposal procedures. The requirements of many washes in an immunometric or hybridization approach are eliminated. The self-contained device does not require special equipment, other than a standard, constant temperature heat block. The low complexity of the device lends itself to "point of care" testing in clinics and physician's offices. The portability of the device provides for "on site" analysis to detect nucleic acid sequences in the areas of forensics, agriculture, environment and the food industry.

Nucleic acid probe technology has developed rapidly in recent years as the scientific community has discovered its value for detection of various diseases, organisms or genetic abnormalities. Amplification techniques have provided the sensitivity to qualitatively determine the presence of even minute quantities of nucleic acid. The drawback to wide spread use of this technology is the possibility of cross contamination of samples since the test is so sensitive. The cost of nucleic acid based testing is high as it requires highly skilled technicians and sophisticated equipment. One method of eliminating the possibility of carry over from one sample to another, is to use a completely enclosed disposable device.

SUMMARY OF INVENTION

This invention is based on a novel concept for a method for detecting specific DNA or RNA sequences. The present invention is defined by a self-contained device integrating nucleic acid extraction, amplification and detection methodologies.

The present invention is a self-contained device that integrates nucleic acid extraction, specific target amplification and detection into a single device, permitting rapid and accurate nucleic acid sequence detection. The present invention is applicable to all nucleic acids and derivatives thereof The present invention is useful to identity specific nucleic acid sequences corresponding to certain diseases or conditions as well as monitoring efficacy in the treatment of contagious diseases, but is not intended to be limited to these uses.

In an embodiment of the invention, the self-contained device comprises a first hollow elongated cylinder with a single closed end and a plurality of chambers therein, a second hollow elongated cylinder positioned contiguously inside the first cylinder capable of relative rotation. Sample is introduced into the second cylinder for extraction. The extracted nucleic acid is bound to a solid phase membrane or silica, and therefore not eluted from the solid phase by the addition of wash buffer. Amplification and labeling takes place in the same cylinder. Finally, the labeled, amplified product is reacted with microparticles conjugated with receptor specific ligands for detection of the target sequence.

In another embodiment of the invention, sample is extracted, amplified and detected in three separate and sequential chambers.

Other features and advantages of the present invention will become apparent from the following detailed description, taken in conjunction with the accompanying figures, that illustrate by way of example, the principles of the instant invention.

The present invention relates generally to a self-contained device integrating nucleic acid extraction, specific target amplification, and detection. This invention relies on the principles of chromatographic nucleic acid extraction from the sample, amplification of specific target nucleic acid sequences resulting in a dual labeled amplification product, ligand-receptor binding, and microparticle technology for detection of amplified nucleic acid. Furthermore, the instant invention may rely on nucleic acid hybridization.

The process according to the present invention is suitable for the determination of all nucleic acid target sequences. The sensitivity and accuracy of this process are improved compared to the processes currently used by those skilled in the art. The invention offers the possibility of contamination free, rapid and reliable determination of the presence of specific amplified target nucleic acids.

BRIEF DESCRIPTION OF THE FIGURES

The file of this patent contains at least one figure executed in color. Copies of this patent with color figure(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

FIG. **1** is a perspective view of a self-contained device integrating nucleic acid extraction, amplification and detection.

FIG. **2** is a schematic of the preferred sealing mechanism, illustrating each of the three device rotational positions: A) closed; B) open; and C) elute.

FIG. **3** is a cross-sectional view of the upper and lower bodies of the device, showing the hinged cover in the open position.

FIG. **4** is a perspective view of the hinged cover and the reaction bead contained within a reaction bead chamber having an integral knife-edge.

FIG. **5** is a cross-sectional view of the aperture section of the second hollow elongated cylinder.

FIG. **6** depicts the relative position of the absorbent pad and strip having microparticles and capture zones.

FIG. **7** depicts a sequential perspective view illustrating the operating sequence of the self-contained device.

FIG. **8** illustrates the reagents and their perspective interaction in the amplification chamber of the device in an SDA strategy.

FIG. **9** depicts reagents and their respective interactions in an alternate SDA strategy.

FIG. **10** depicts the reagents and their respective interactions in a cycling probe assay.

FIG. **11** illustrates the detection results of isothermal amplification and detection with bifunctionally labeled amplified target sequence using strand displacement assay.

FIG. **12** shows the detection results of a lateral flow assay.

FIG. **13** shows the detection results of an alternate lateral flow.

FIG. **14** depicts a NASBA strategy.

FIG. **15** shows the results of detection by amplification with a single labeled primer followed by hybridization with a probe containing a single label.

REFERENCE NUMERALS IN DRAWINGS

1 First hollow elongated cylinder
2 Second hollow elongated cylinder
3 Hinged cover
6 Index pin
7 Index notch
9 Absorbent pad
10 Strip
11 Reaction bead
12 Reaction bead chamber
13 Aperture
14 Living hinge
15 Sealing lip
16 Reservoir
17 Solid surface
18 Knife-edge
19 Foil or foil/polymer membrane
20 Detection chamber
21 Transparent viewing window
22 Porous membrane
23 Silica slurry
24 Colored microparticles
25 Capture zone for target sequence
26 Capture zone for control sequence

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

The present invention provides a method of detecting an amplified target nucleic acid sequence that is present in a sample. It is recognized by those skilled in the art that assays for a broad range of target nucleic acid sequences present in a sample may be performed in accordance with the present invention. Samples may include biological samples derived from agriculture sources, bacterial and viral sources, and from human or other animal sources, as well as other samples such as waste or drinking water, agricultural products, processed foodstuff, air, etc. Examples include blood, stool, sputum, mucus, serum, urine, saliva, teardrop, a biopsy sample, an histological tissue sample, a tissue culture product, an agricultural product, waste or drinking water, foodstuff, air, etc. The present invention is useful for the detection of nucleic acid sequences indicative of genetic defects or contagious diseases.

The following definitions will be helpful in understanding the specification and claims. The definitions provided herein should be borne in mind when these terms are used in the following examples and throughout the instant application.

As used herein, the term "target" nucleic acid molecule refers to the nucleic acid molecule that is amplified by the presented methods. The "target" molecule can be purified, partially purified, or present in an unpurified state in the sample.

As used in this invention, the term "amplification" refers to a "template-dependent process" that results in an increase in the concentration of a nucleic acid sequence relative to its initial concentration. A "template-dependent process" is defined as a process that involves the "template-dependent extension" of a "primer" molecule. A "primer" molecule refers to a sequence of nucleic acid that is complementary to a portion of the target or control sequence and may or may not be labeled with a hapten. A "template dependent extension" refers to nucleic acid synthesis of RNA or DNA wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the rules of complementary base pairing of the target nucleic acid and the primers.

The present invention relates to the extraction and amplification of nucleic acids in a chamber of a self-contained device, followed by detection in a another chamber, and collection of waste in, yet, another chamber. The reaction chambers are functionally distinct, sequential and compact. Said chambers deliver precise volumes, dispense reagents and collect waste. All of this occurs in a completely self-contained device with simple, fool proof directions for use as described below.

As illustrated in FIG. **1**, an extraction, amplification and detection device consists of a first hollow elongated cylinder **1** having one closed end and an integrally-molded cover **3** hinged to the opposing, open end and a second hollow elongated cylinder **2** that is positioned contiguously inside the first cylinder **1** and capable of relative rotation. The preferred embodiment of the second cylinder **2** is a tapered cylinder terminating with an aperture **13** having a sealing lip **15**. The first cylinder **1** further consists of 2 chambers: a reservoir **16** and a detection chamber **20**, said detection chamber further consisting of a pad **9** and a strip **10**.

The bulk of the device is composed of a material that does not facilitate binding of nucleic acids and proteins. The preferred material is heat and cold resistant material which is light weight, rigid and sturdy. The preferred size is compact enough to fit into conventional size heat blocks, however, the size may be scaled up or down, accordingly. The preferred embodiment inserts the device into a constant temperature environment, such as a heat block, allowing the reactions to proceed at the preferred conditions of constant temperature.

When sample is introduced into the device, nucleic acid extraction and amplification takes place in the second cylinder **2**, said first hollow elongated cylinder **2** containing the detection chamber **20** having a means for detection. The reservoir **16** collects the lysis buffer used in the extraction process and subsequent washes.

The second cylinder **2** rotates relative to the first cylinder **1** and locks into position A, position B or position C. At the tapered end of the second cylinder **2**, an aperture **13** having a sealing lip **15** enables the second cylinder **2** to engage with

either the detection chamber **20** or reservoir **16**. The first cylinder **1** contains two chambers, the reservoir **16** and the detection chamber **20**. The hinged cover **3** has one indexing pin **6** used for locking the second cylinder **2** in positions A, B and C. The second cylinder **2** is closed to the reservoir **16** in the A, or closed, position. In the B, or open, position, the second cylinder **2** allows flow to the reservoir **16**. In the C, or elute, position, amplified nucleic acid target and control are able to wick into the detection chamber **20**. The hinged cover **3** also contains a reaction bead **11** within a reaction bead chamber **12**. This bead **11** contains the reaction enzymes and other reagents required for the amplification step. The second cylinder **2** contains three notches **7** for indexing with the indexing pin **6** and locking the relative rotation of cylinders **1** and **2**.

In position A, the second cylinder **2** is sealed, allowing for the extraction step and the amplification step to take place. In position B, the second cylinder **2** is such that the opening in the second cylinder **2** is not sealed and is over the reservoir **16**. In position C, the second cylinder **2** is rotated such that the second cylinder **2** is not sealed and the opening is over an absorbent pad **9** located in the detection chamber **20**. The absorbent pad **9** collects the amplified product and wicks the product onto a strip **10** of nylon, nitrocellulose or other suitable material. The strip **10** contains colored microparticles **24** and capture zones for the target **25** and the control **26** sequences. The detection chamber **20** contains a transparent viewing window **21** for observing the results of the reaction.

FIG. **2** illustrates the preferred embodiment of the sealing mechanism of the device disclosed herein. In open position A, the second cylinder **2** is sealed by a sealing lip **15**. The sealing lip **15** is composed of a flexible material that can be compressed when in contact with a solid surface **17** at the top of the first cylinder **1**. In close position B, rotation of the second cylinder **2** relative to the first cylinder **1** allow the contents of the second cylinder **2** to flow into the reservoir **16** through a porous membrane **22** in the bottom of the second cylinder **2**. In this position, the sealing lip **15** is extended beyond the plane of compressi ,n and allows fluid to flow into the reservoir **16**. The second cylinder **2** can be rotated relative to the first cylinder **1** into elute position C. In this position, the sealing lip **15** is again extended beyond the plane of compression over an opening containing an absorbent pad **9** and a strip **10** of membrane use for the detection step.

A cross-section of the upper **1** and lower **2** body of the device and the hinged cover **3** in the open position is illustrated in FIG. **3**. The index pin **6** is located on the hinged cover **3**. Three index notches **7** are located on the second cylinder **2**. The reaction bead **11** contains lyophilized enzymes and reagents for the amplification reaction. The hinged cover **3** contains a knife-edge **18**, which when sufficient pressure is applied thereto punctures a foil membrane **19** releasing the reaction bead **11** into the second cylinder **2**, as shown in FIG. **4**.

A cross-section of the bottom of the second cylinder **2** is illustrated in FIG. **5**. The sealing lip **15** contains a porous membrane **22** that binds the extracted nucleic acids or a porous membrane **22** that holds a silica slurry **23** in the second cylinder **2**. A strip **10** containing a region with immobilized colored microparticle **24** and two capture zones **25**, **26** is depicted in FIG. **6**. The microparticles **24** are coated with a receptor that is specific to the target and the control sequence. Target sequence capture zone **25** contains receptors specific for haptens on the target sequence and control sequence capture zone **26** contains receptors specific for haptens on the control sequence.

The following examples serve to explain and illustrate the present invention. Said examples are not to be construed as limiting of the invention in anyway. Various modifications are possible within the scope of the invention.

EXAMPLE 1

Sample Flow Through the Preferred Embodiment of a Self-Contained Device

The preferred embodiment of the device disclosed herein is defined by two hollow elongated cylinders, a first cylinder having a closed end, as illustrated in FIG. **1**, for the extraction, amplification and detection of nucleic acid sequences. In the close position A, sample is introduced into the second cylinder **2**. The second cylinder **2** contains dry lysing reagents for extraction of nucleic acids. The sample provides the liquid that resuspends the lysing reagents. After a brief incubation period with the cover **3** closed, the second cylinder **2** is rotated into open position B. The extracted nucleic acid remains in the upper chamber bound to the porous membrane **22** or the silica slurry **23**, while the liquid flows into the reservoir **16**. In this position, several washes of buffer or water follow. Next, the second cylinder **2** is rotated into close position A such that the second cylinder **2** is sealed, water is added and the cover closed. When sufficient pressure is applied to the hinged cover **3**, the reaction bead **11** is released from the reaction bead chamber **12** and added to the second cylinder **2** by breaking the foil membrane **19** with the knife-edge **18**. The reaction bead **11** carries the enzymes necessary for amplification, which are resuspended in the water and amplification takes place on the membrane **22** or silica slurry **23** containing the extracted nucleic acids. After an appropriate incubation period, the second cylinder **2** is rotated relative to the first cylinder **1** into elute position C. The amplification reaction mixture is able to enter the detection chamber **20** as it is absorbed onto the pad **9**. When the pad **9** absorbs a sufficient amount of liquid, the reaction mixture is wicked up the strip **10**. On the strip, the colored microparticles **24** bind to haptens resulting from the amplification reaction and travel to the capture zone on the membrane where they form a visible line of detection if the target sequence is present and for the control sequence. The line of detection is viewed from the transparent viewing window **21**. See FIG. **7**.

The second cylinder **2** has a capacity of 0.001 to 25 ml. Sample is whole blood, sputum, serum, plasma, urine, fecal matter, a tissue, part of an organ or any other source that may contain the target nucleic sequence. Sample is from humans, plants or animals and may be environmental in nature.

The method and apparatus disclosed herein provides for extremely rapid, economical nucleic acid detection. Further, this self-contained device significantly reduces the risk of cross contamination in that neither amplification reagents nor amplicons are manipulated. The minimal additional instrumentation required, a standard heat block, and simplicity of the protocol, enable the test to be performed easily, anywhere and with a minimum amount of technical experience.

EXAMPLE 2

Microparticle Selection

The preferred microparticles utilized in this invention are composed of polymeric materials such as latex polyethylene, polypropylene, polymethylmethacrylate or polystyrene. However, a variety of other synthetic or natural

materials may also be used in the preparation of the microparticles, for example, silicates, paramagnetic particles and colloidal gold. The usual form of microparticles possesses surface sulfate charge groups that can be modified by the introduction of functional groups such as hydroxyl, carboxyl, amine and carboxylate groups. The functional groups are used to bind a wide variety of ligands and receptors to the microparticles. These groups are selected on the basis of their ability to facilitate binding with the selected member of the ligand-receptor pair, either by covalent binding or adsorption. The preferred method of attachment of the receptor to the microparticles is covalent binding.

The size of the microparticles used in this invention is selected to optimize the binding and detection of the labeled amplicons. Microparticles are available in a size range of 0.01–10.0 μm in diameter. The preferred diameter for this embodiment of the invention is a range of 0.01–1.0 μm, specifically not excluding the use of either larger or smaller microparticles as appropriately determined. The microparticles are activated with a suitable receptor for binding to the target ligand. The preferred microparticle in the present invention is composed of latex containing a colored dye.

In the present invention, microparticle bound receptors are specific for discreet haptens located on the ends of amplified nucleic acid sequences. The receptors must be capable of binding to their specific binding partner (hapten) and, further, changing the derivatized haptens from the preferred biotin and digoxigenin necessitates a change in the receptors. Conjugation of the receptors to the microparticle is accomplished by covalent binding or, in appropriate cases, by adsorption of the receptor onto the surface of the microparticle. Techniques for the adsorption or covalent binding of receptors to microparticles are well known in the art and require no further explanation.

In order to prepare the anti-digoxigenin coated microparticles, 0.25–1.0 mg/ml of anti-digoxigenin Fab is incubated with a suspension containing a final concentration of 1.0% microparticles/ml. The microparticles and digoxigenin Fab are allowed to react for 15 minutes prior to treatment with activating agent for covalent binding. The microparticles are treated with EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiamide) at a final concentration of 0–2.5 mM. The Fab and microparticles are mixed and incubated at room temperature for one hour. Unbound Fab is removed by successive washes and the coated microparticles are resuspended in storage buffer.

Lateral flow assays are performed on nylon or nitrocellulose membranes spotted with capture zones of 1.0 μl streptavidin at concentrations between 0.0 and 1.0 mg/ml.

EXAMPLE 3

Amplification

The present invention employs a variety of different enzymes to accomplish amplification of the target nucleic acid sequence, for example, polymerases and ligases. Polymerases are defined by their function of incorporating nucleoside triphosphates to extend a 3' hydroxyl terminus of a "primer molecule." As used herein, a "primer" is an oligonucleotide, that when hybridized to a target nucleic acid molecule, possesses a 3' hydroxyl terminus that can be extended by a polymerase and a hapten label at or near the 5' terminus. For a general discussion concerning polymerases, see Watson, J. D. et al., (1987) *Molecular Biology of the Gene*, 4th Ed., W. A. Benjamin, Inc., Menlo Park, Calif. Examples of polymerases that can be used in accordance with the methods described herein include, but are not limited to, *E. coli* DNA polymerase I, the large proteolytic fragment of *E. coli* polymerase I, commonly known as "Klenow" polymerase, Taq-polymerase, T7 polymerase, T4 polymerase, T5 polymerase and reverse transcriptase. The general principles and conditions for amplification of nucleic acids using polymerase chain reaction, as discussed supra, are well known in the art.

EXAMPLE 4

Isothermal Amplification Approach to Detection with Labeled Amplified Target Sequence Using NASBA

The preferred embodiment for amplification using this invention is an isothermal reaction such as NASBA (U.S. Pat. No. 5,130,238, specifically incorporated herein) or strand displacement assay (SDA)(Walker et al. (1992) PNAS 89:392, specifically incorporated herein). The primary product of the NASBA reaction is single strand RNA. The NASBA reaction utilizes a primer containing a T7 polymerase promoter. Following T7 transcription, up to 100 copies of target RNA are produced. These copies are the same sequence as the original target RNA. They serve as templates, thus, starting the cycle again and resulting in up to a billion fold amplification of the original template.

In order to incorporate NASBA into the device disclosed herein, probes that allow the formation of a bifunctionally haptenized amplification product have been designed. For NASBA there are two possible strategies: 1) design amplification primers that are haptenized; and 2) use two haptenized capture oligos which bind to the product RNA. See, for example, FIGS. **8** and **9**. The model system chosen is to the HIV POL gene.

In the instant NASBA haptenization strategy, the T7NASFAM haptenization primer, containing a T7 transcriptase promoter and an attached fluorescein, binds to the target RNA. A reverse transcriptase transcribes a DNA copy of the RNA, as illustrated in example B of FIG. **14**. The original RNA strand is digested by RNase H. A reverse haptenization primer, P2NASBIO with attached biotin binds to the antisense DNA and is extended by the DNA polymerase activity of the reverse transcriptase. The haptenized primers are as follows:

```
T7NASFAM (T7-PROMOTER PRIMER):
5'-FLUORESCEIN
-AATTCTAATACGACTCACTATAGGGTGCTATGTCACTTCCCCTTGGTTCTCT-3' SEQ ID NO:1

P2NASBIO (REVERSE PRIMER):
5'BIOTIN-AGTGGGGGGACATCAAGCAGCCATGCAAA-3'                SEQ ID NO:2
```

The resulting double-stranded bi-haptenization DNA intermediate is illustrated in example D of FIG. **14**. This complex gives signal in lateral flow or slide agglutination.

T7 RNA polymerase binds to the promoter region to manufacture many copies of a minus-sense RNA, as shown in example F of FIG. **14**. This RNA contributes to the manufacture of the DNA intermediate by similar means. Two capture oligos, each having one hapten of either fluorescein or biotin, bind to the (−)sense RNAs giving bifunctional haptenized complexes. These complexes give signal in lateral flow or slide agglutination. The haptenized capture oligos designed to bind to the minus-sense RNA product are:

```
5C(-)NASBA:
5'FLUORESCEIN-TGGCCTGGTGCAATAGGCCC-3'  SEQ ID NO:3

3C(-)NASBA:
5'CCCATTCTGCAGCTTCCTCA-BIOTIN-3'       SEQ ID NO:4
```

EXAMPLE 5

Isothermal Amplification Approach to Detection with Bifunctionally Labeled Amplified Target Sequence Using Strand Displacement Assay

The instant strand displacement assay (SDA) is an example of an isothermal amplification that can be detected by using microparticles and bifunctionally labeled product. SDA technology is described in U.S. Pat. No. 5,455,166 to Becton Dickinson and Company, specifically incorporated herein.

SDA is isothermal amplification based on the ability of a restriction enzyme to nick the unmodified strand of a hemiphosphorothioate from of its recognition site and the ability of DNA polymerase to initiate replication at the nick and displace the downstream non-template strand. Primers containing recognition sites for the nicking restriction enzyme bind to opposite strands of target DNA at positions flanking the sequence to be amplified. The target fragment is exponentially amplified by coupling sense and antisense reactions in which strands displaced from the sense reaction serve as a target for the antisense reaction and vice versa.

This set of experiments is conducted with composite extension primers that are labeled with biotin, fam or digoxigenin. Bumper primers are the same sequence as provided by Becton Dickinson and Company (Franklin Lakes, N.J.). The sequences of the target, the bumper primer and the composite extension primer are as follows:

```
Bumper Primers:
B1: 5'-CGATCGAGCAAGCCA                                  SEQ ID NO:5

B2: 5'-CGAGCCGCTCGCTGA                                  SEQ ID NO:6

Composite extension primers:
S1: 5'-fam/dig-ACCGCATCGAATGCATGTCTCGGGTAAGGCGTACTCGACC  SEQ ID NO:7

S2: 5'-biotin-CGATTCCGCTCCAGACTTCTCGGGTGTACTGAGATCCCCT   SEQ ID NO:8

Target sequence:
5'TGGACCCGCCAACAAGAAGGCGTACTCGACCTGAAAGACGTTATCCACCAT    SEQ ID NO:9

ACGGATAGGGGATCTCAGTACACATCGATCCGGTTCAGCG
```

The reaction is set up per the thermophilic Strand Displacement Amplification (tSDA) protocol developed by Becton Dickinson and Company. The target organism is *Mycobacterium tuberculosis*. For pilot studies, an artificial target template consisting of the 91nt sequence of the *M tuberculosis* genome, defined by the Becton Dickinson outer (bumper) primers, is used. Amplification conditions used are identical to those used by Becton Dickinson for tSDA.

Membrane used for this procedure is nitrocellulose, purchased from Millipore Corporation, Bedford, Mass. A stripe of streptavidin at a concentration of 1 mg/ml is applied at a rate of 1 μl/cm via a linear reagent striper (IVEK Corporation, No. Springfield, Vt.) 1 cm from the bottom edge of the membrane. After application of the streptavidin, the membrane is allowed to dry and then blocked for non-specific binding by 0.5% casein in 100 mM Tris, pH 7.4. The membranes are washed twice with water (ddH_2O) and allowed to dry. Next, 3 μl of anti-S1 (complementary to S1 without the biotin label) and/or S2 primer (complementary to S2 without the dig or fam label) is spotted onto a second membrane. This membrane is sandwiched onto the first membrane in order to capture free primers that compete with the product for the microparticles or streptavidin capture zone. The microparticles are prepared as outlined supra in Example 2 with either anti-digoxigenin Fab or anti-fam monoclonal IgG. The microparticles are diluted 1:2 with a 35% sucrose solution and 3 μl applied directly to the membrane and dried.

The reaction product (10 μl) is added to 45 μl SDA buffer, then applied (50 μl) to the previously striped membrane. Application of the sample requires the bifunctionally labeled product and the competing primers to pass through the anti-primer coated membrane and the dried microparticles. When the target is present, there is a visible line on the membrane. When the target is not present, there is absence of a visible line. The results of one such experiment are shown in FIG. **11**.

EXAMPLE 6

Inhibition Assay: Loss of Visible Signal on Lateral Flow Membrane

Cycling probe technology involves a nucleic acid probe that incorporates DNA-RNA-DNA sequences designed to hybridize with the target sequences. See, for example, FIG. **10**. The probes are bifunctionally labeled with biotin and fam. If the probes hybridize with the target generating double stranded nucleic acid, RNase H in the reaction buffer cleaves the probes. This cleavage results in loss of signal when applied to a membrane containing a capture zone of streptavidin and anti-fam coated, colored microparticles. If the target is not present, there is a visible line on the membrane.

The specific probe and target employed in the instant example have been designed by ID Biomedical Corporation for use in detecting *Mycobacterium tuberculosis*. The probe is a chimeric construct containing both DNA and RNA sequences with labels on the 5' (fam) and the 3' (biotin) ends of the DNA portion of the probe. The binding of the probe to a single strand of target generates double stranded nucleic acid which is cleaved with RNase H, thus, eliminating the

bifunctionality of the probe. The sequence of the probe is described below:

SEQ ID NO:10

FARK2S3B probe
5'-fam AAA GAT GT agag GGT ACA GA-3'biotin
(lower case indicates deoxyribonucleoside bases)

SEQ ID NO:11

The sequence of the target is described below:
ARK2-T synthetic target
5'-AAT CTG TAC CCT CTA CAT CTT TAA-3'

The reaction is completed following the protocol provided by ID Biomedical Corporation. Membrane used for this procedure is nitrocellulose, purchased from Millipore Corporation, Bedford, Mass. A stripe of streptavidin at a concentration of 1 mg/ml is applied at a rate of 1 μl/cm via a linear reagent striper (IVEK Corporation, No. Springfield, Vt.) 1 cm from the bottom edge of the membrane. After application of the streptavidin, the membrane is allowed to dry and then blocked for non-specific binding by 0.5% casein in 100 mM Tris, pH 7.4. The membranes are washed twice with water (ddH_2O) and allowed to dry. The microparticles used are prepared as outlined supra in Example 2, replacing anti-digoxigenin Fab with anti-fam monoclonal IgG.

The reaction product (10 μl) is added to 5 μl of 0.1% anti-fam coated microparticles (0.1%) and 35 μl of water, then applied (50 μl) to the previously striped membrane. The binding of the probe to the target followed by cleavage of the probe by RNase H, results in loss of the bifunctionality of the probe. When the target is present, the absence of a visible line on the membrane exists. When the target is not present, the bifunctionally labeled probe is able to bind the anti-fam coated microparticles and the streptavidin bound to the membrane, resulting in a visible line. The results of one such experiment are shown in FIG. **12**.

With amplification, certain specimens are inhibitory to the amplification reaction providing false-negative results. To avoid this problem, a positive control—a control nucleic acid with primer recognition sequences attached to a totally irrelevant nucleic acid sequence—is incorporated. This positive control primer is a component of the nucleic acid extraction reagents in second cylinder of the device, thus, controlling for sample extraction and delivery as well as detecting amplification failure. The preferred embodiment of the positive control is a lambda DNA sequence. The control nucleic acid is extracted and amplified along with the target nucleic acid and is detected by a line of immobile anti-digoxigenin beads on the detection solid phase.

The target oligonucleotide primer and the control oligonucleotide primer used in this invention contain at least one hapten as label which does not participate in the priming reaction. The hapten is bound to at least one position of the nucleic acid primer. For the derivatization of nucleic acid primers, various methods can be employed. See, Sambrook supra. The incorporation of the hapten can take place enzymatically, chemically or photochemically. The hapten can be derivatized directly to the 5' end of the primer or contain a bridge 1 to 30 atoms long. In the preferred embodiment, the bridge is linear. However, in an alternate embodiment, the bridge consists of a branched chain with a hapten molecule on at least one of the chain ends. By means of the presence of several hapten molecules on the ends of a branched chain, the detection sensitivity is increased. The preferred haptens for the present invention are biotin and digoxigenin, however, other haptens having a receptor as specific binding agent available are suitable, for example, steroids, halogens and 2,4 dinitrophenyl.

EXAMPLE 7

Detection of Bifunctionally Labeled Amplified Product

Membrane used for this procedure is nitrocellulose, purchased from Millipore Corporation, Bedford, Mass. A stripe of streptavidin at a concentration of 1 mg/ml is applied at a rate of 1 μl/cm via a linear reagent striper (IVEK Corporation, No. Springfield, Vt.) 1 cm from the bottom edge of the membrane. After application of the streptavidin, the membrane is allowed to dry and then blocked for non-specific binding by 0.5% casein in 100 mM Tris, pH 7.4. The membranes are washed twice with water (ddH_2O) and allowed to dry.

The amplification product is added to the membranes with colored receptor coated beads at dilutions of 0.001–1.0% microparticles/ml. This mixture is allowed to wick up the membrane. Positive reactions result in a colored line where the capture material is applied. Amplification reactions without the target sequence added to the reaction serve as negative controls. The results of one of these experiments are illustrated in FIG. **13**.

If the target and control nucleic acid sequence are present, the receptor bound microparticles interact with hapten(s) to capture the amplified nucleic acid. The result, a line of dyed particles visible on the membrane for the target and for the control nucleic acids. If the target is not present, the dyed particles are not captured and are not visible. When the result of the analysis is negative, the control nucleic acid sequences must be visible indicating that the extraction and amplification were performed correctly.

EXAMPLE 8

Detection by Amplification with a Single Labeled Primer Followed by Hybridization with a Probe That Contains a Single Label

The target nucleic acid sequence is amplified by PCR using 200–1000 mM primer concentration. GeneAmp EZ rTth RNA PCR kit (Perkin Elmer Corp., Alameda, Calif.) and 10^6 copies/ml of the target HIV RNA sequence. Forty PCR cycles, each cycle being 60° C. for 15 minutes, 95° C. for 15 seconds, and 55° C. for 60 seconds, are run.

The sequences of the primers is as follows:

SEQ ID NO:12

SK38 Dig Primer
5'-DIG ATA ATC CAC CTA TCC CAG TAG GAG AAA T-3'

SEQ ID NO:13

SK39 Primer
5'-TT TGG TCC TTG TCT TAT GTC CAG AAT GC-3'

Specific PCR reaction conditions are described below:

Reagent	Final conc.
5X EZ Buffer	1X
$Mn(OAc)_2$	3 mM
rTth polymerase	5 U
dntp's	240 μM each
SK38	1 μM
SK39	1 μM

rTth DNA Polymerase from Perkin Elmer N808-0097

The SK38 Dig—SK39 amplicon (5 μl) is incubated with 5 μl of 25 μM (125 pmol) SK39 biotin at 95° C. for 1 minute, and then 55° C. for 1 minute. The amplicon bound to the anti-digoxigenin microparticles wicks through the membrane to the streptavidin line and is captured by the inter-

action of biotin and streptavidin. The result is a visible line of colored microparticles.

In the negative control, the procedure is performed as described above, but without the addition of the target sequence. Without the presence of the target sequence in the amplification reaction, the bifunctionally labeled amplicon is not generated and the visible line of detection is not present. The results of one such experiment are shown in FIG. 15.

EXAMPLE 9

Alternate Embodiment of a Self-Contained Device

Sample is introduced into an extraction chamber for extraction of nucleic acid. This chamber incorporates a nucleic acid extraction/solid phase nucleic acid binding protocol providing a rapid method of nucleic acid purification. The preferred extraction method makes use of chaotropic agents such as guanidine isothiocynate to disrupt the cell membranes and extract the nucleic acid. Proteins are degraded by proteinases. The extracted nucleic acid binds to a solid phase membrane in the extraction chamber. The nucleic acid is eluted from the solid phase by the addition of elution buffer. The design of a fitting between the solid phase membrane and a seal prevents waste from entering the amplification chamber.

After the sample is added to the extraction chamber, a supply assembly unit locks onto the top of a processor assembly unit by connecting a first and a second fitting. Following a 10–15 minute incubation allowing nucleic acid extraction, the first of four plungers is depressed. Air in a compartment forces the extraction mixture past the solid phase membrane binding the nucleic acid. The filtrate is collected in a waste chamber. Depression of the second plunger forces a wash buffer stored in a wash buffer compartment across the solid phase membrane and filtrate passes to the waste chamber. The seal located directly below the solid phase membrane is disposed at an angle to aid in efficient collection of the waste. Depressing the third plunger forces air stored in a compartment across the solid phase membrane, insuring that all of the wash buffer is removed. The processor assembly unit twists, simultaneously breaking the seal and closing off a waste chamber conduit. Depressing the f urth plunger delivers an elution buffer stored in a compartment for elution of the nucleic acid from the solid phase and delivers a volume of nucleic acid into an amplification chamber.

In the alternate embodiment, the amplification chamber contains the reagents for amplification and hybridization. In additional alternative embodiments, reagents for amplification and hybridization are in separate chambers. This process is characterized in that the sample is treated, after extraction, with two distinct labeled oligonucleotides primers. The sequence of the first primer is complementary to a partial sequence of a strand of the target nucleic acid and is labeled with hapten, for example, biotin. The sequence of the second primer is complementary to a partial sequence of the control nucleic acid and labeled with a second hapten, for example, digoxigenin. Either primer may contain a promoter region. Subjecting the mixture to amplification, preferably isothermal amplification, results in hapten labeled target and control nucleic acid. These labeled, amplified nucleic acid sequences react with oligonucleotides conjugated to microparticles of suitable color and diameter for detection. The microparticles are conjugated with an oligo specific for binding nucleic acid sequence on the target. The microparticles are conjugated with an oligo specific for binding nucleic acid on the control. The resulting microparticles, bound by hybridization to the amplicons, are detected in the detection chamber.

EXAMPLE 10

Extraction of Nucleic Acids with Quanidinium Thiocyanate onto Glass (Silica Dioxide) and Subsequent Amplification Without Elution from Silica Dioxide

A column was constructed using Ansys 0.4 mm membrane as filter to contain the silica dioxide and a syringe apparatus to pull buffer through the column in approximately 15 seconds. 50 µl serum, 2 µl SiO2 (0.5 mg/µl), and 450 µl GuSCN lysis buffer are mixed by vortexing and then incubated at room temperature for 10 minutes. The specific lysis buffer for the instant set of experiments contains 14.71 g GuSCN (4M final), 0.61 ml "Triton X-100", 5.5 ml 0.2M EDTA pH 8.0 and is q.s. to 31.11 ml with 0.1M Tris-HCl pH 6.4. The silica dioxide is washed twice with 500 µl 70% ETOH.

Next, the filter with SiO2 is removed from the column and the SiO2 washed off of the membrane using 20 µl water (ddH_2O). 5 µl silica dioxide slurry is added to a PCR reaction using standard protocol for HIV model system, as detailed supra in Example 8.

The instant invention provides a rapid, simple and accurate method of detecting amplified target nucleic acid sequences with a self-contained device. Sensitivity and specificity of the assay are based on labeling of the target, by incorporating label or by subsequent hybridization of labeled probed, during the amplification process. The method does not require costly and sophisticated equipment or specially trained personnel, nor does it pose any health hazard.

While the above description contains many specificities, these should not be construed as limitations on the scope of the invention, but rather an exemplification of the preferred embodiment thereof. Many other variations are possible, such as amplifying several target samples in the same reaction mixture, utilizing newly discovered polymerases and ligases, etc. Thus, the scope of the invention should be determined by the appended claims and their legal equivalents, rather than by the example given.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 13

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 52 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATTCTAATA CGACTCACTA TAGGGTGCTA TGTCACTTCC 40

CCTTGGTTCT CT 52

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGTGGGGGGA CATCAAGCAG CCATGCAAA 29

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGGCCTGGTG CAATAGGCCC 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCATTCTGC AGCTTCCTCA 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGATCGAGCA AGCCA 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGAGCCGCTC GCTGA 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

REFERENCES

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

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